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Future management and possible treatment of halitosis

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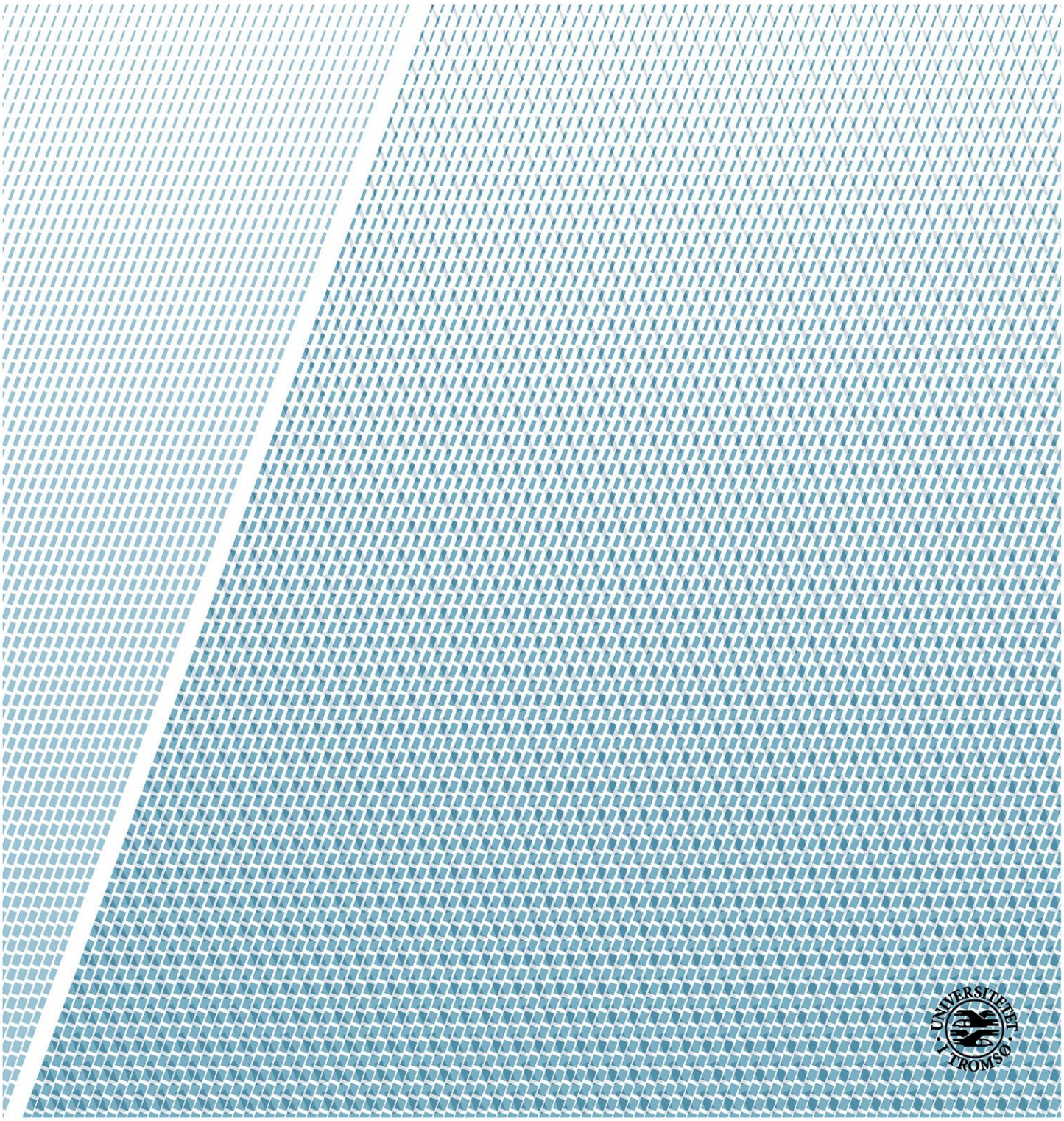


Acknowledgement

I want to give a big heads up for my supervisor Raafat El-Gewely, which has spent a lot of effort and time in guiding me through this thesis. He has open my horizons in seeing the possibilities in modern drug discovery and how dentistry can benefit in this field.

For the past two years I have gained knowledge about the importance of having the right people around when things in life aren't going as smooth as one might hope. The right people will support you, they will listen, they will care, they will never see you any different from others and when you lose the belief in yourself they will strengthen your faith in gaining it back. With this I really want to give a big thank you to these people, but especially big thanks and hug to Siril Nergård and Lene M. Rasmussen.

Contents

1. Abstract

Halitosis is caused by the release of volatile sulfur compounds. Today, we find endless products in the market that provide long lasting, fresh and minty breath. But how do these products work against halitosis, and a more important question is how effective are they?

To discover a possible management, treatment or even cure of halitosis we need to know the causes. Here we focused on oral bacterial species as serious causes that are known to cause halitosis. These bacteria are from different species, genera and families, but they all produce volatile sulfur compounds (VSC). These VSC are produced by different enzymes. Inhibiting the activity of these enzymes by a non-toxic compound could help prevent or cure halitosis. Focusing on these related enzymes as targets for inhibitors would be of prime importance for halitosis.

Respective enzymes are proteins encoded by related genes in the genomes of these bacterial species. The main enzymes of focus are: L-cysteine desulfhydrase, methionine gamma-lyase and L-methionine-alpha-deamino-gamma-mercaptomethane-lyase. Comparing the amino acid sequence of the proteins as well as the nucleotide sequence of the corresponding genes is made to study the degree of relatedness (homology) among these enzymes of the different bacteria. One aim of this study was to predict how one discovered inhibitor could work or not on the other enzymes. A homology study of known enzymes; L-cysteine desulfhydrase, methionine gamma-lyase and L-methionine-alpha-deamino-gamma-mercaptomethane-lyase (METase) that are involved in the production of volatile sulfur compounds is conducted. We have looked into the amino acid sequence of these enzymes and the sequence of their coding genes and found the oral bacteria that have high degree of sequence homology for these three enzymes. Similar enzymes to the target enzymes were found in *Fusobacterium* sp. oral taxon 370, *Fusobacterium periodonticum* and many subspecies of *Fusobacterium nucleatum*. Knowing that many oral bacteria that causes halitosis contains similar enzymes; these enzymes could the targets for drug discovery for halitosis treatment.

2. Introduction

2.1 History of halitosis

The problem of halitosis to man has existed for thousands of years. The word halitosis originates from Latin, where "*halitus*" meaning breath and the ending "*osis*" in medical terms, describes a pathologic alteration. (3).

Cultural indifferences have addressed this problem in their own way; Islamic teaching stresses the use of a special wooden stick called the miswaak/ miswak (Fig.1 left) (1, 4). This traditional brushing stick is made of small brushes prepared from small twigs prepared from the tree *Salvadora persica* L (Fig. 1, right) belonging to the *Salvadoraceae* family. Miswak is generally obtained from any slim woody part of the tree. (16).

Study (Balto et al, 2012) has found that *S. persica* extract is somewhat comparable to other oral disinfectants and anti-plaque agents, such as triclosan and chlorhexidine gluconate, if used at sufficiently high concentrations. The clinical interest of *S. persica* arises from a number of mechanisms, including its acidic and antimicrobial properties. By the isolation of the active ingredient from *S. persica*, Wolinsky and Sote (14) found antimicrobial activity against various Gram positive and Gram negative bacteria.

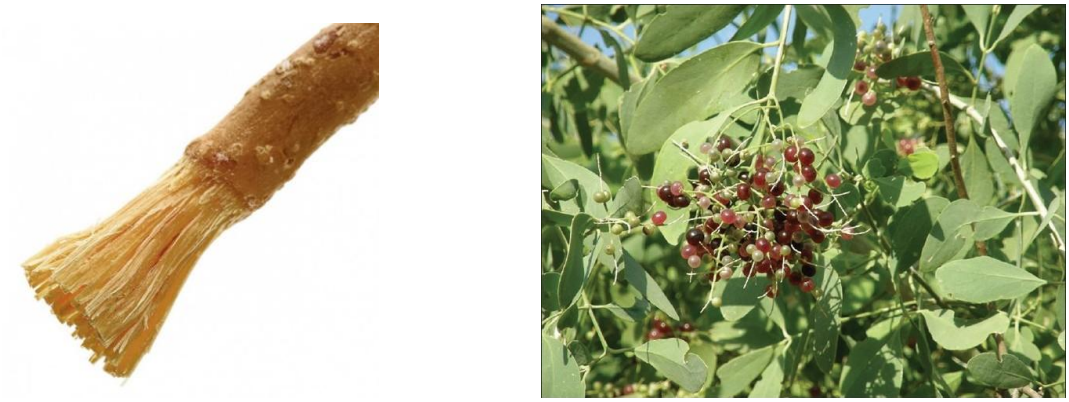


Fig. 1: Left: Miswaak/ Miswak http://muslimvillage.com/2012/03/17/20703/miswak-a-great-sunnah-and-a-healthy-habit/ from the plant species *Salvadora persica* (right) http://www.jpbsonline.org/viewimage.asp?img=JPharmBioallSci_2011_3_1_113_76488_f2.jpg

S.persica (Miswak sticks) possess plaque inhibiting and antibacterial properties against several types of cariogenic bacteria frequently found in the oral cavity. Vahabi et al. (16) confirm that the antimicrobial effect of alcoholic extract of *Salvadora persica* is believed to be due to its content in chlorides, tannins, trimety-lamine salvadorine, nitrate, thiocynate and sulfur. A pharmacological study revealed that the antiplaque activity of *S. persica* was comparable with chlorexhidine gluconate (16).

Further, Talmud suggests peppercorns, the Bible (Genesis) mentions labdanum (mastic, Fig.1, left), a resin derived from the tree *Pistacia lentiscus* (Fig.2, right) that has been used in Mediterranean countries, and which is thought to have been used as chewing gum. Other natural or folk remedies can be found in the literature including parsley (Italy), cloves (Iraq), guava peels (Thailand), anise seeds (Far East), cinnamon (Brazil) and eggshells (China) (1, 2).


Fig 2. : Left: Mastic gum, (plant resin), (http://en.wikipedia.org/wiki/Mastic the resin from three *Pistacia lentiscus* tree (right) (http://www.botanical-online.com/fotos9.htm)

Mastic gum has been previously shown to demonstrate antimicrobial activity. A previous study shows the strong antimalodorous activity of mastic gum in a salivary incubation assay and demonstrated anti-microbial activities, VSC conversion properties and proteolysis inhibition abilities. This suggested that this natural medicine might serve as effective agents in oral malodor treatment (15).

Several antiseptic agents including chlorhexidine, cetyl pyridinium chloride, fluorides and phenol derivatives have been used widely in dentistry to inhibit bacterial growth (19). Nevertheless, dental scientists have still been searching for new applications of therapeutic drugs to prevent or treat dental plaque-related diseases. Studies have confirmed an antibacterial effect of mastic gum on mutans streptococci. In analyzing mastic gum is seen to have the main constituents of leaves of mastic tree (*P. lentiscus*) which contain terpinen-4-ol and a-terpineol. These constituents are believed to be active compounds of many essential oils, and particularly tea tree oil (19).

2.2 Causes of halitosis

The causes of halitosis can be divided into:

I) Systemic/ extra-oral
II) Intraoral

Extra-oral conditions that cause halitosis and their prevalence (%) are shown below:

> Ear, nose, throat associated 10%
> Gastrointestinal/ endocrinological 5%
> Halitophobia, psychiatrical, psychological problems
> (5)

The epidemiology studies amongst the prevalence of halitosis and intraoral causes are limited. Although extra-oral conditions can give rise to halitosis, it is the intraoral causes that are of importance when talking about halitosis, where insufficient dental hygiene, periodontitis or tongue coating accounts for 85% of the cases of halitosis (4).

Intraoral conditions that cause halitosis are shown below (4, 5):

> Insufficient dental hygiene
> Periodontitis
> Tongue coating
> Cleaning of dentures
> Dry mouth

Oral malodor can be affected by the intake of food and drinks, which can either dry the mouth, such as alcohol-containing liquids and cigarettes. Furthermore, dairy products are known to break down in the mouth leading to the release of amino acids that are rich in sulfur. This is also true for onion and garlic that also contain high concentrations of sulfur, which can pass through the lining of intestine into the bloodstream, and subsequently be released into the lungs and then exhaled. Smoking not only raises the concentration of volatile compounds in the mouth and lungs, but also further aggravates the situation because of its drying effect on the oral mucosa (2).

2.3 Current methods in reducing halitosis

With the many anti-halitosis products available today, they all have different approaches in trying to either mask or try to solve this problem (9).

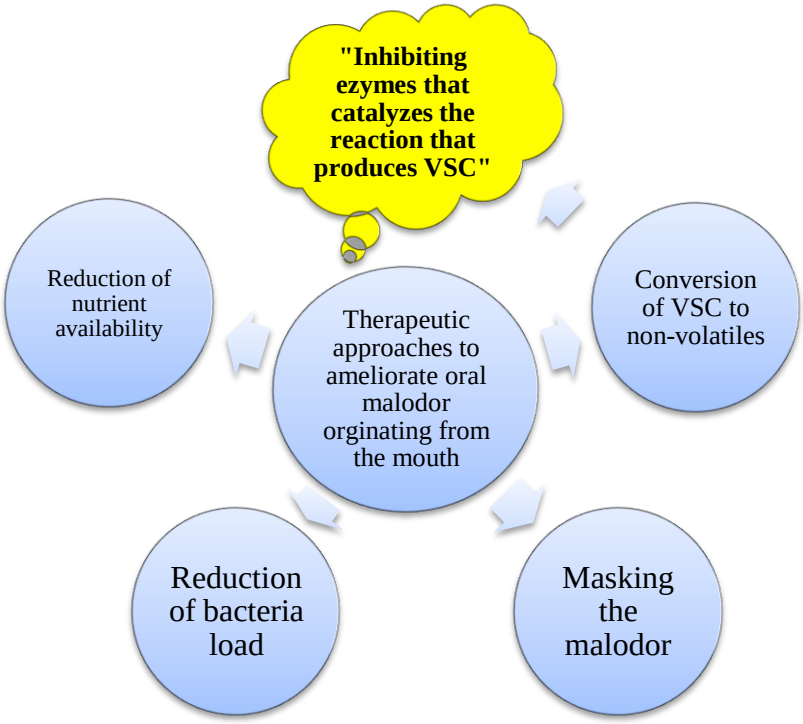


Fig. 2: Shows different method and approaches in reducing oral malodor. VSC: volatile sulfur compounds.

The different approaches are interesting regarding the effectiveness in the actual ameliorating effect. Masking the malodor with, say, mouthwashes, strong flavor chewing gums will only give a short term effect, but will not reduce the problem, whilst reducing the bacteria load

might disrupt the normal flora in the oral cavity, an example is using chlorhexidine-based products, which will give rise to oral candida infections (Table 1). Another approach in reducing the bacteria load is mechanically; tooth brushing and scraping the tongue, but the duration of the effects varies from 15-100 min (5).

Mouthwash products added with the "secret ingredient" claim to reduce halitosis, but have their limitations, either time wise or with unfavorable side effects. These secret ingredients, or better known as the active ingredient, are the key of the anti-halitosis effect. A list of active ingredients found in anti-halitosis products is shown in Table1.

Table1. Active ingredients with promising anti-halitosis effect (5, 6, 7, 8, and 20).

Active Ingredient	Chlorhexidine (0.2%, 0.12%)	Essential Oils	Triclosan	Cetylpyridinium chloride (CFC)	Zinc salts	Chlorine dioxide
How it works	A strong oxidizing molecule, attacks the bacterial cell membrane causing leakage or precipitation of the cellular contents (6).	Disrupt cell wall and inhibiting enzyme activity. Inhibits bacterial multiplication and extracts endotoxins from Gram negative species (6).	Phenolic agent with broad-spectrum antibacterial activity that disrupt bacterial cytoplasmic membrane by blocking fatty acid biosynthesis (7).	Binds non-specifically to charged protein and modifies surface tension of the bacterial cell wall, thus leading to cell wall leakage and affecting cell metabolism (20).	Metal ions oxidize the thiol groups in the precursors of volatile sulfur-containing compounds (5). Inhibit bacterial cysteine proteases (20).	Oxidizes amino acids methionine and cysteine (9).
Other benefits in the oral cavity	Antibacterial Antiplaque Antigingivitis	Antibacterial Antiplaque Antigingivitis Antiinflammatorical (5).	Antimicrobial Antiplaque	Reduce plaque accumulation and gingival inflammation	Antibacterial	Antibacterial
Side effects	Irritation to oral mucosa, tooth and tongue staining, burning sensation, altered taste perception (5).	Side effects are not verified	Side effects are not verified	Burning mouth sensation, staining of tongue and teeth ulceration (8).	Side effects are not verified	Side effects are not verified

Recently, epigallocatechin gallate (EGCg), a polyphenolic catechin from tea (*Camellia sinensis*), has been suggested as an alternative agent for halitosis management. EGCg has the ability to inhibit the growth of *P.gingivalis,* a halitosis-associated bacterium due to the expression of *mgl* gene. This gene is coding for L-methionine-α-deamino-γ-mercaptomethane-lyase, responsible for methyl mercaptan (CH_3SH) production by oral anaerobes. This enzyme is also inhibited by EGCg (29).

In this thesis, the focus will be on the future perspective of treating halitosis by drug developed in analogous way to modern methods of drug discovery. That is by identifying the causing target protein / enzyme, target validation, lead compounds discovery, lead compound optimization, preclinical and clinical studies.
Ironically, such studies have not been made before although enzymes from key oral bacteria have been implicated in producing volatile sulfur compounds (VSC).

3. Origin of halitosis

In general, halitosis most often results from the microbial degradation of oral organic substrates, either from food, saliva or gingival fluid. Where during this degradation process volatile sulfur compounds (VSC) are formed causing our bad breath problem (5).

Malodor is due mainly to putrefactive actions of bacteria on endogeneous or exogeneous proteins and peptides. The major offending compounds are hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH), and to a lesser extent, dimethylmercaptan (CH_3SSCH_3). These sulfides are produced mainly from substrates; cysteine and methionine that are found in saliva, gingival cervical fluid and tongue coating debris (10, 20).

The oral bacteria that are able to produce VSC; methyl mercaptan and hydrogen sulfide are shown in Table 2. Common with these bacteria is that they all are gram-negative anaerobes.

Table 2. Bacteria that produces VSC (4)

Hydrogen sulfide from cysteine	*Peptosteptococcus anaerobius*
	Micros prevotii
	Eubacterium limosum
	Bacteroides spp.
	Centipedia periodontii
Hydrogen sulfide from serum	*Prevotella intermedia*
	Prevotella loescheii
	Porphyromonas gingivalis (BANA positive)
	Treponema denticola (BANA positive)
	Selenomonas artermidis
Methyl mercaptan from methionine	*Fusobacterium nucleatum*
	Fusobacterium periodonticum
	Eubacterium spp.
	Bacteroides spp.
Methyl mercaptan from serum	*Treponema denticola* (BANA positive)
	Porphyromonas gingivalis (BANA positive)
	Porphyromonas endodontalis
Others	*Prevotella melaninogenica*
	Tanerella forsythia
	Eikenella corrodens
	Solobacterium moorei
	Treponema forsythensis
	Centipeda periodontii
	Atopobium parvulum

There are quite a few oral bacteria that use sulfur containing amino acids for their metabolism fuel. The oral bacteria mentioned in the literature that are most likely to cause oral malodor are Gram-negative bacteria species, including:

Treponema denticola
Porphyromonas gingivalis
Porphyromonas endodontalis
Prevotella intermedia
Bacteroides loescheii
Enterobacteriace- ae
Tannerella forsythia
Centipeda periodontii
Eikenella corrodens
Fusobacterium nucleatum
Solobacterium moorei
(5, 20)

4. Enzymes and reactions leading to the release of VSC

As mentioned earlier, substrates that produce VSC are S-amino acids cysteine, and methionine which transforms into their corresponding product hydrogen sulfide (H_2S), methyl mercaptan (CH_3SH), and to a lesser extent, dimethylmercaptan (CH_3SSCH_3) (11, 21)

Methyl mercaptan is a highly toxic compound and is thought to play an important role in periodontal disease (21)

Mentioned in the literature are catalyzing enzymes that converts sulfur containing amino acids into products of volatile sulfur compounds. From this chemical reactions can be deducted and shown below as equation I-III (11, 23, 24).

Equation I) $\text{L-cysteine} \xrightarrow{\text{L-cysteine desulfhydrase}} H_2S + \text{pyruvate} + \text{ammonia}$ (23)

Equation II) $\text{Cysteine} \xrightarrow{\text{L-methione-}\gamma\text{-lyase}} H_2S + \text{pyruvate} + \text{ammonia}$ (11)

Equation III) $\text{L-methionine} \xrightarrow{\text{METase}} \alpha\text{-ketobyturate} + \text{methyl mercaptan} + \text{ammonia}$ (24)

From the equations the enzymes catalyzing the chemical reaction in the production of VSC are L-methionine-γ-lyase, L-cysteine desulfhydrase and METase (L-methionine- alpha-deamino-gamma-mercaptomethane-lyase) (11)

MET-ase has been detected in anaerobic oral bacteria, such as *Porphyromonas gingivalis*, *Fusobacterium nucleatum* and *Treponema denticola*. The encoding gene is mgl. (12)

5. Homology study of key enzymes

The genetic sequence and amino acid sequence of each of the three enzymes; L-methionine-γ-lyase, L-cysteine desulfhydrase, METase (L-methionine- alpha-deamino-gamma-mercaptomethane-lyase) are to be found, further we are going to see which other oral bacteria contain each of these enzymes or enzymes with similar amino acid sequence.

If the outcome results show many of the mentioned oral bacteria in Table 2, this enzyme is of significant in halitosis production and inhibiting this enzyme, in theory, will give a good anti-halitosis effect. The tool used is BLAST (Basic local alignment search tool). The nucleotide sequences are compared against all sequenced bacterial species found in human.

A recent review published in June 2013 in the Journal of Dental Research links oral bacteria to extra-oral infections and inflammation processes (13). The author summarizes with a table connecting extra-oral infections to oral species, which includes *Fusobacteria nucleatum*. In the review Han and Wang link *F.nucleatum* to cardiovascular disease, adverse pregnancy outcomes, rheumatoid arthritis, inflammatory bowel disease, meningitis or brain abscesses, lung, liver, or splenic abscesses and even appendicitis and colorectal cancer.

This is of interest to this thesis in the sense that *F. nucleatum* is one of the main bacteria that are able to produce the enzymes catalyzing the reaction of sulfur gases. Again, gaining more information to the genetic level will help us one step closer in making a cure to the oral and systemic diseases (13).

The enzymes of interest are as follow:

I) L-cysteine desulfhydrase
II) L-methionine-gamma-lyase
III) L-methionine-alpha-deamino-gamma-mercaptomethane-lyase

The protein BLAST will be preferred in the homology study below; this is because even with small differences in the nucleotide sequences, several triplet nucleotides can give rise to the same amino acid.

The ten first BLAST hit will be included as well as oral bacteria that are found further down the result list, using percentage identity to compare how much alike different enzymes are to each other.

Table 3. Halitosis related enzymes; their encoding genes and their produced volatile sulfur compounds.

Enzyme	Encoding gene	Volatile sulfur compound
L-cysteine desulfhydrase	*lcs*	H_2S
L-methionine-gamma-lyase	*megL*	H_2S
L-methionine-alpha-deamino-gamma-mercaptomethane-lyase	*mgl*	Methyl mercaptan

5.1 Enzyme 1: L-cysteine desulfhydrase

Query: L-cysteine desulfhydrase
Source (organism): *Aggregatibacter actinomycetemcomitans*

Gene sequence

```
   1 atgacatact atccagcaga gccgttccga atcaaaagtg ttgaaccggt ttccatttta
  61 ccgaaagcag aacgcgaaaa agcaatgaaa gaagcgggat ataatacctt cttacttgat
 121 tcaaaagacg tatatatcga tctcttaacc gatagcggta ccaatgccat gagtgatcgt
 181 caatgggcag gtattatgct gggagatgaa gcttacgccg gtagtagaaa cttctatcat
 241 ctgcaagaaa ccgtacaaga actcttcggt ttcaaacata tcgttccgac ccaccaagga
 301 cgtggtgcgg aaaatatcct ttcccgtatt gctatcaaac cgggacaata tgtgccgggc
 361 aatatgtatt tcaccacaac ccgttatcac caagaagcca acggcggtat tttctacgac
 421 attattcgtg atgaagccca tgatgcgaca ttagacgtgc cattcaaagg tgatattgat
 481 ctgaaaaaac tggaaaacct gattaatgaa aaaggggcgg aaaacatcgc ttatgtatgt
 541 ttagcggtca ccgtgaacct cgccggcggt caaccggttt ccatcgccaa catgaaagcc
 601 gtgcgcgaac tcactgctaa acacggcatc aaagtgttct acgacgccac ccgttgtgtt
 661 gaaaatgcgt acttcattaa agaacaggaa aaaggctacc aagatcgctc cattaaatcc
 721 attattcacg aaatgttcag ttatgccgac ggttgcacca tgagtggtaa aaaagactgc
 781 ttaaccaata tcggcggttt cttatgtatg aacgatgaag aattgttcat gaaagccaaa
 841 gaattggtag tggtgtttga aggtatgccg tcttatggcg gtatggcggg tcgtgatatg
 901 gaagccatgg caatcggttt gaaagaagcc acccaagaag aatacattga acaccgtgtg
 961 aaacaagtac gttacctcgg cgaaaaatta aaagccgccg gtgtaccgat tgttgaaccg
1021 attggtggtc atgccgtatt cttggatgcc cgtcgtttct gcccgcatct gaaacaagag
1081 gaagatttcc cggcacaagc cttggcggcg gcaatctata tcgaatgtgg cgtgcgtacc
1141 atggaacggg gtattatatc cgccggtcgt gatgtaaaaa ccggtgaaaa ccaccgtccg
1201 aaacttgaaa ccgtgcgtat caccattcct cgccgcgttt atacctatac ccatatggat
1261 ttagtagctg acggtattat ccgtctgttt aaacataaag gagatattaa aggtcttcgt
1321 ttcgtgtatg aaccgaaaca actccgtttc ttcactgcac gttttgaaca aaagtag

//
```

Amino acid sequence

```
   1 mtyypaepfr iksvepvsil pkaerekamk eagyntflld skdvyidllt dsgtnamsdr
  61 qwagimlgde ayagsrnfyh lqetvqelfg fkhivpthqg rgaenilsri aikpgqyvpg
 121 nmyftttryh qeanggifyd iirdeahdat ldvpfkgdid lkklenline kgaeniayvc
 181 lavtvnlagg qpvsianmka vreltakhgi kvfydatrcv enayfikeqe kgyqdrsiks
 241 iihemfsyad gctmsgkkdc ltniggflcm ndeelfmkak elvvvfegmp syggmagrdm
 301 eamaiglkea tqeeyiehrv kqvrylgekl kaagvpivep igghavflda rrfcphlkqe
 361 edfpaqalaa aiyiecgvrt mergiisagr dvktgenhrp kletvritip rrvytythmd
 421 lvadgiirlf khkgdikglr fvyepkqlrf ftarfeqk
```

BLAST result

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics Distance tree of results Multiple alignment

Description	Max score	Total score	Query cover	E value	Ident
L-cysteine desulfhydrase [Aggregatibacter actinomycetemcomitans] >gb|EHK91116.1| tyrosine phenol-lyase [Aggregatibacter actinomycetemcomitans Rh	952	952	100%	0.0	100%
L-cysteine desulfhydrase [Aggregatibacter actinomycetemcomitans] >gb|EGY33256.1| tyrosine phenol-lyase [Aggregatibacter actinomycetemcomitans ser	947	947	100%	0.0	99%
L-cysteine desulfhydrase [Aggregatibacter actinomycetemcomitans] >gb|EGY39764.1| tyrosine phenol-lyase [Aggregatibacter actinomycetemcomitans ser	943	943	100%	0.0	99%
tyrosine phenol-lyase [Aggregatibacter actinomycetemcomitans D7S-1] >ref|WP_005544068.1| L-cysteine desulfhydrase [Aggregatibacter actinomycetemc	941	941	100%	0.0	99%
L-cysteine desulfhydrase [Aggregatibacter actinomycetemcomitans] >gb|EGY42226.1| tyrosine phenol-lyase [Aggregatibacter actinomycetemcomitans ser	941	941	100%	0.0	98%
L-cysteine desulfhydrase [Aggregatibacter actinomycetemcomitans] >gb|EGY42550.1| tyrosine phenol-lyase [Aggregatibacter actinomycetemcomitans ser	937	937	100%	0.0	98%
tyrosine phenol-lyase [Aggregatibacter actinomycetemcomitans] >gb|ELT53913.1| tyrosine phenol-lyase [Aggregatibacter actinomycetemcomitans serotyp	937	937	100%	0.0	98%
tyrosine phenol-lyase [Aggregatibacter actinomycetemcomitans ANH9381] >ref|WP_005566879.1| L-cysteine desulfhydrase [Aggregatibacter actinomycete	936	936	100%	0.0	98%
tyrosine phenol-lyase [Aggregatibacter aphrophilus NJ8700] >ref|WP_005701517.1| L-cysteine desulfhydrase [Aggregatibacter aphrophilus] >gb|ACS9656	934	934	100%	0.0	97%
tyrosine phenol-lyase [Aggregatibacter actinomycetemcomitans D11S-1] >ref|WP_005548314.1| L-cysteine desulfhydrase [Aggregatibacter actinomyceten	932	932	100%	0.0	98%
L-cysteine desulfhydrase [Pasteurella pneumotropica]	921	921	100%	0.0	96%
L-cysteine desulfhydrase [Haemophilus parainfluenzae] >gb|EGC73023.1| tyrosine phenol-lyase [Haemophilus parainfluenzae ATCC 33392]	920	920	100%	0.0	96%
tryptophanase/L-cysteine desulfhydrase, PLP-dependent [Haemophilus parainfluenzae T3T1] >ref|WP_014064489.1| L-cysteine desulfhydrase [Haemoph	920	920	100%	0.0	95%
L-cysteine desulfhydrase [Fusobacterium russii]	823	823	99%	0.0	85%
tyrosine phenol-lyase [Fusobacterium nucleatum subsp. animalis ATCC 51191]	822	822	98%	0.0	86%
tyrosine phenol-lyase [Fusobacterium nucleatum] >gb|ERT32121.1| tyrosine phenol-lyase [Fusobacterium nucleatum CTI-5]	821	821	98%	0.0	85%
L-cysteine desulfhydrase [Fusobacterium nucleatum] >gb|EDK87683.1| tyrosine phenol-lyase [Fusobacterium nucleatum subsp. polymorphum ATCC 10	821	821	98%	0.0	86%
L-cysteine desulfhydrase [Fusobacterium periodonticum] >gb|EFE87107.1| tyrosine phenol-lyase [Fusobacterium periodonticum ATCC 33693]	821	821	98%	0.0	85%
L-cysteine desulfhydrase [Fusobacterium nucleatum] >gb|EJU08456.1| tyrosine phenol-lyase [Fusobacterium nucleatum ChDC F128]	820	820	98%	0.0	85%
L-cysteine desulfhydrase [Fusobacterium nucleatum] >gb|EEO40791.1| tyrosine phenol-lyase [Fusobacterium nucleatum subsp. vincentii 4_1_13]	820	820	98%	0.0	85%
tyrosine phenol-lyase [Fusobacterium nucleatum subsp. animalis 4_8] >ref|WP_005910458.1| L-cysteine desulfhydrase [Fusobacterium] >gb|EEO43797.	819	819	98%	0.0	85%

Beyond the ten first hits

tyrosine phenol-lyase [Fusobacterium nucleatum subsp. vincentii 3_1_36A2] >ref|WP_005889817.1| L-cysteine desulfhydrase [Fusobacterium nucleatum	819	819	98%	0.0	85%
L-cysteine desulfhydrase [Fusobacterium nucleatum] >gb|EFD81838.1| tyrosine phenol-lyase [Fusobacterium nucleatum subsp. animalis D11]	820	820	98%	0.0	85%
L-cysteine desulfhydrase [Fusobacterium periodonticum] >gb|EEO38196.1| tyrosine phenol-lyase [Fusobacterium periodonticum 2_1_31] >gb|EKA92858.	819	819	98%	0.0	85%
tyrosine phenol-lyase [Fusobacterium nucleatum subsp. nucleatum ATCC 25586] >ref|WP_011015962.1| L-cysteine desulfhydrase [Fusobacterium nucle	816	816	98%	0.0	85%
L-cysteine desulfhydrase [Fusobacterium nucleatum] >gb|EFG95136.1| tyrosine phenol-lyase [Fusobacterium nucleatum subsp. nucleatum ATCC 23726]	816	816	98%	0.0	85%
L-cysteine desulfhydrase [Citrobacter youngae] >gb|EFE07355.1| tyrosine phenol-lyase [Citrobacter youngae ATCC 29220]	809	809	98%	0.0	83%
L-cysteine desulfhydrase [Citrobacter freundii] >gb|EHL84013.1| tyrosine phenol-lyase [Citrobacter freundii 4_7_47CFAA]	808	808	98%	0.0	82%
tyrosine phenol-lyase [Citrobacter sp. KTE32] >sp|P31012.1|TPL_ESCIN RecName: Full=Tyrosine phenol-lyase; AltName: Full=Beta-tyrosinase >pir||S263	808	808	98%	0.0	82%
tyrosine phenol-lyase [Citrobacter] >gb|EOQ51380.1| tyrosine phenol-lyase [Citrobacter sp. KTE151]	807	807	98%	0.0	82%
L-cysteine desulfhydrase [Citrobacter] >gb|EJF23075.1| Tyrosine phenol-lyase [Citrobacter sp. A1] >gb|EKU35933.1| tyrosine phenol-lyase [Citrobacter sp.	807	807	98%	0.0	82%
L-cysteine desulfhydrase [Citrobacter sp. 30_2] >gb|EEH94863.1| tyrosine phenol-lyase [Citrobacter sp. 30_2]	806	806	98%	0.0	82%
L-cysteine desulfhydrase [Citrobacter] >sp|P31013.1|TPL_CITFR RecName: Full=Tyrosine phenol-lyase; AltName: Full=Beta-tyrosinase >pdb|2EZ2|A Cha	805	805	98%	0.0	82%
Chain A, Tyrosine Phenol-lyase From Citrobacter Intermedius Complex With 3-(4'- Hydroxyphenyl)propionic Acid, Pyridoxal-5'-phosphate And Cs+ Ion >pdb	805	805	98%	0.0	82%
Tryptophanase [Escherichia coli ISC11]	805	805	98%	0.0	82%
Tryptophanase [Morganella morganii subsp. morganii KT] >ref|WP_004241032.1| L-cysteine desulfhydrase [Morganella morganii] >gb|AGG31995.1| Trypt	805	805	98%	0.0	83%
Chain A, Y71f Mutant Of Tyrosine Phenol-Lyase From Citrobacter Freundii In Complex With Quinonoid Intermediate Formed With 3-Fluoro-L-Tyrosine >pdb	804	804	98%	0.0	82%
Chain A, Holo Tyrosine Phenol-Lyase From Citrobacter Freundii At Ph 8.0 >pdb|2EZ1|B Chain B, Holo Tyrosine Phenol-Lyase From Citrobacter Freundii At	803	803	98%	0.0	82%
Chain A, F448h Mutant Of Tyrosine Phenol-Lyase From Citrobacter Freundii In Complex With Quinonoid Intermediate Formed With 3-Fluoro-L-Tyrosine >p	803	803	98%	0.0	82%
Chain A, D214a Mutant Of Tyrosine Phenol-Lyase From Citrobacter Freundii >pdb|2YHK|B Chain B, D214a Mutant Of Tyrosine Phenol-Lyase From Citroba	803	803	98%	0.0	82%
L-cysteine desulfhydrase [Treponema denticola] >gb|EGC76365.1| tyrosine phenol-lyase [Treponema denticola F0402] >gb|EMB20780.1| tyrosine phenol-	802	802	99%	0.0	84%
tyrosine phenol-lyase [Treponema denticola] >gb|EMB22816.1| tyrosine phenol-lyase [Treponema denticola SP37] >gb|EPF34476.1| tyrosine phenol-lyase	802	802	99%	0.0	84%
tyrosine phenol-lyase [Treponema denticola ATCC 35405] >ref|WP_002682416.1| L-cysteine desulfhydrase [Treponema denticola] >gb|AAS11607.1| tyros	801	801	99%	0.0	83%
tyrosine phenol-lyase [Treponema denticola] >gb|EMB27830.1| tyrosine phenol-lyase [Treponema denticola MYR-T] >gb|EMB28715.1| tyrosine phenol-lyas	801	801	99%	0.0	83%

Table 4. Oral bacterial species with similar enzyme activity and sequence to L-cysteine desulfhydrase produced from *Aggregatibacter actinomycetemcomitans*, including oral species beyond the ten first hits.

Oral bacteria	**Identity in amino acid sequence to L-cysteine desulfhydrase in *Aggregatibacter actinomycetemcomitans* (%)**
Fusobacterium nucleatum subsp. animalis	86
Fusobacterium nucleatum	85
Fusobacterium periodonticum	85
Fusobacterium nucleatum supsp. vincentii	85
Treponema denticola	84

Query: L-cysteine desulfhydrase
Source (organism): *Fusobacterium nucleatum subsp.polymorphum* ATCC 10953

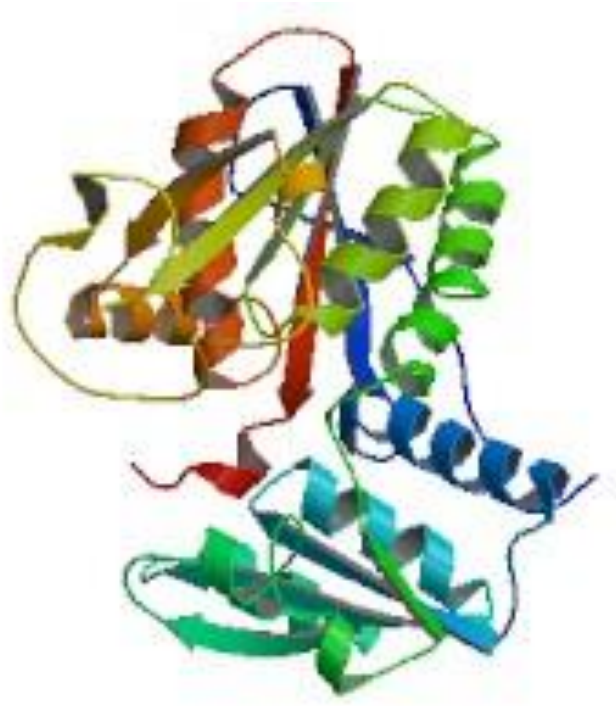
Gene sequence

```
        1 aaaatttaat ttattatatt tcaatattat tctttaaaaa ataagaactc tatatttttt
       61 ttaatgagtt cttttatttt ttttctttta gttatacaat taagttgaaa ataaagtttt
      121 ataggaggat ttttatgtta gcaaattctg taattgattt aattgggaac accccattag
      181 taaaaattaa taatattaat acttttggaa atgaaatata tgtaaaacta gaaggttcaa
      241 atcctggtag aagtacaaaa gacagaattg ccttaaaaat gattgaagaa gctgaaaaag
      301 aaggtttaat tgataaagat actgttatta tagaagctac aagtggaaat acaggaattg
      361 ggcttgctat gatatgtgca gttaaaaact ataagttaaa gattgttatg cctgatacta
      421 tgagtgttga aagaattcaa cttatgagag cctatggaac tgaagttata cttactgatg
      481 gttcttttgg aatgaaagct tgtttagaaa aattagaaga acttaaaaaa caagaaaaga
      541 aatattttat tcctaaccaa tttactaatg taaataatcc aaaagctcac tatgaaacta
      601 cagctgagga aattttaaga gatatggata ataaagttga tgtatatatt tgtggaacag
      661 gaacaggagg aagtttttct ggaactgcta aaaaattaaa agaaaaatta cctaatatta
      721 aaacttaccc cgttgaacct gcgtcatctc ctttactttc aaagggatat ataggtccac
      781 ataaaattca aggtatggga atgagtatag gtggtatacc agttgtctac gatggtagtt
      841 tagctgatgg aattttagtt tgtgaagatg atgaagcctt taaaatgatg agagaattaa
      901 gctttaaaga aggtatctta gctgggattt caacaggtgc tactctaaaa gcagctcttg
      961 attattcaaa agaaaatgct aataaaagtt taagaatagt tgttctttct actgactcag
     1021 gagaaaaata tctatctagt tctcatggct tataaaaaat attccaagaa gttgc
//
```

Amino acid sequence

```
        1 mlansvidli gntplvkinn intfgneiyv klegsnpgrs tkdrialkmi eeaekeglid
       61 kdtviieats gntgiglami cavknyklki vmpdtmsver iqlmraygte viltdgsfgm
      121 kaclekleel kkqekkyfip nqftnvnnpk ahyettaeei lrdmdnkvdv yicgtgtggs
      181 fsgtakklke klpniktypv epasspllsk gyigphkiqg mgmsiggipv vydgsladgi
      241 lvceddeafk mmrelsfkeg ilagistgat lkaaldyske nankslrivv lstdsgekyl
      301 ssshgl
//
```

Protein structure of L-cysteine desulfhydrase with the same amino acid sequence as above retrieved at MODBASE, a database of comparative protein structure models is shown below (Fig. 3).



(Fig. 3: protein structure of L-cysteine desulfhydrase, http://modbase.compbio.ucsf.edu/modbasecgi/model_details.cgi?queryfile=1379269881_6868&searchmode=default&displaymode=moddetail&referer=yes&snpflag=&)

Blast result:

Top first ten bacteria with similar amino acid sequence to L-cysteine desulfhydrase found in *fusobacterium nucleatum subsp.polymorphum.*)

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics Distance tree of results Multiple alignment

Description	Max score	Total score	Query cover	E value	Ident
cysteine synthase [Fusobacterium nucleatum] >dbj|BAC02912.1| L-cysteine desulfhydrase [Fusobacterium nucleatum] >gb|EDK88376.1| cysteine synth	620	620	100%	0.0	100%
cysteine synthase [Fusobacterium nucleatum] >gb|EJU07268.1| cysteine synthase [Fusobacterium nucleatum ChDC F128]	612	612	100%	0.0	98%
cysteine synthase [Fusobacterium nucleatum] >gb|EEO40077.1| cysteine synthase A [Fusobacterium nucleatum subsp. vincentii 4_1_13]	563	563	100%	0.0	94%
cysteine synthase [Fusobacterium nucleatum] >gb|EJG09653.1| cysteine synthase [Fusobacterium nucleatum subsp. fusiforme ATCC 51190]	552	552	98%	0.0	93%
cysteine synthase [Fusobacterium nucleatum subsp. nucleatum ATCC 25586] >ref|WP_011016993.1| cysteine synthase [Fusobacterium nucleatum] >g	550	550	98%	0.0	93%
cysteine synthase [Fusobacterium nucleatum] >gb|EFG95837.1| cysteine synthase A [Fusobacterium nucleatum subsp. nucleatum ATCC 23726]	545	545	98%	0.0	92%
cysteine synthase [Fusobacterium periodonticum] >gb|EFE85510.1| cysteine synthase A [Fusobacterium periodonticum ATCC 33693]	535	535	99%	0.0	94%
cysteine synthase [Fusobacterium nucleatum subsp. vincentii 3_1_36A2] >ref|WP_008799582.1| cysteine synthase [Fusobacterium nucleatum] >gb|EE	535	535	100%	0.0	94%
cysteine synthase [Fusobacterium periodonticum] >gb|EEO37375.2| cysteine synthase A [Fusobacterium periodonticum 2_1_31]	533	533	99%	0.0	93%
cysteine synthase [Fusobacterium periodonticum] >gb|EFG29011.1| cysteine synthase A [Fusobacterium periodonticum 1_1_41FAA]	533	533	99%	0.0	93%
cysteine synthase [Fusobacterium nucleatum] >gb|EFG34280.1| cysteine synthase A [Fusobacterium nucleatum subsp. vincentii 3_1_27]	529	529	98%	0.0	94%

The ten first hits show many sub species of *fusobacterium nucleatum* that produces similar enzyme as the query species *fusobacterium nucleatum subsp.polymorphum.*

Number six down the list we find a 94% identity hit with cysteine synthase from *fusobacterium periodonticum* mentioned in table 2, which also produces methyl mercaptan from methionine.

Using a more sensitive protein-protein search called “Delta Blast”, which allow us to identify similarities and gaps of the amino acid sequences.

Here, we conduct a comparison of L-cysteine desulfhydrase from *fusobacterium nucleatum subsp.polymorphum* and the oral bacteria *fusobacterium periodonticum,* which produces cysteine synthase.

Delta Blast result

Query ID	gi|21715911|dbj|BAC02912.1|	**Subject ID**	gi|492819376|ref|WP_005975774.1|
Description	L-cysteine desulfhydrase [Fusobacterium nucleatum]	**Description**	cysteine synthase [Fusobacterium periodonticum]
Molecule type	amino acid	**Molecule type**	amino acid
Query Length	306	**Subject Length**	306
		Program	BLASTP 2.2.28+ ▷Citation

Range 1: 1 to 304 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
535 bits(1379)	0.0	Compositional matrix adjust.	285/304(94%)	299/304(98%)	0/304(0%)

```
Query  1    MLANSVIDLIGNTPLVKINNINTFGNEIYVKLEGSNPGRSTKDRIALKMIEEAEKEGLID  60
            MLANSVIDLIGNTPLVKINNI+TFGNEIY+KLEGSNPGRSTKDRIALKMIEEAEKEGLID
Sbjct  1    MLANSVIDLIGNTPLVKINNIDTFGNEIYIKLEGSNPGRSTKDRIALKMIEEAEKEGLID  60

Query  61   KDTVIIEATSGNTGIGLAMICAVKNYKLKIVMPDTMSVERIQLMRAYGTEVILTDGSFGM  120
            KDTVIIEATSGNTGIGLAMICA+KNYKLKIVMP+TMSVERIQLMRAYGTEVILTDGS GM
Sbjct  61   KDTVIIEATSGNTGIGLAMICAIKNYKLKIVMPNTMSVERIQLMRAYGTEVILTDGSLGM  120

Query  121  KACLEKLEELKKQEKKYFIPNQFTNVNNPKAHYETTAEEILRDMDNKVDVYICGTGTGGS  180
            KACL+KLEELKK+EKKYFIPNQFTN NNPKAHYE TAEEILRDMDNKVDVYICGTGTGGS
Sbjct  121  KACLDKLEELKKEEKKYFIPNQFTNPNNPKAHYENTAEEILRDMDNKVDVYICGTGTGGS  180

Query  181  FSGTAKKLKEKLPNIKTYPVEPASSPLLSKGYIGPHKIQGMGMSIGGIPVVYDGSLADGI  240
            FSGTAKKLKEKLPNIKT+PVEPASSPLLSKGYIGPHKIQGMGMSIGGIPVVYDG+LADGI
Sbjct  181  FSGTAKKLKEKLPNIKTFPVEPASSPLLSKGYIGPHKIQGMGMSIGGIPVVYDGTLADGI  240

Query  241  LVCEDDEAFKMMRELSFKEGILAGISTGATLKAALDYSKENANKSLRIVVLSTDSGEKYL  300
            LVC+D++AFKMMRELSFKEGILAGISTGAT KAALDYSKENANK LRIVVLSTDSGEKYL
Sbjct  241  LVCDDEDAFKMMRELSFKEGILAGISTGATFKAALDYSKENANKGLRIVVLSTDSGEKYL  300

Query  301  SSSH  304
            S+++
Sbjct  301  SNAY  304
```

Fig. 4: showing the two comparing subjects; query = L-cysteine desulfhydrase and subject 1= cysteine synthase Even if the enzyme name is not the same; L-cysteine-desulfhydrase vs. cysteine synthase, both these enzymes gives the same product: hydrogen sulfide. The amino acid sequence of both enzymes shows great similarities (94%), but because of the great variation of bacteria DNA there are many ways for bacteria to get to this specific enzyme.

Another organism that produces L-cysteine desulfhydrase is *Streptococcus anginosus*, the amino acid sequence are analyzed using BLAST (25).

Query: L-cysteine desulfhydrase
Source (organism): *Streptococcus anginosus*

Amino acid sequence

```
  1 mrkynfqtap nrlshhtykw ketetdpqll pawiadmdfe vmpevkqaih dyaeqlvygy
 61 tyasdellqa vldweksehq ysfdkedivf vegvvpaisi aiqaftkegd avlinspvyp
121 pfarsvrlnn rklvsnslke englfqidfe qlekdivenn vklyllcsph npggriwere
181 vlekighlcq khqvilvsde ihqdltlfgh ehvsfntisp dfkefalvls satktfniag
241 tknsyaiien pslraqfkrr qlannhhevs slgyiateta yrygkpwlva lkdvleeniq
301 favdyfakea prlkvmkpqg tyliwldfsd ygltddelft llhdqakvil nrgsdygkeg
361 elharlniat pkplveeick rivhclpq
```

BLAST result

Sequences producing significant alignments:

Select: All None Selected:0

Alignments | Download | GenPept | Graphics | Distance tree of results | Multiple alignment

Description	Max score	Total score	Query cover	E value	Ident
L-cysteine desulfhydrase [Streptococcus anginosus]	808	808	100%	0.0	100%
cysteine desulfhydrase [Streptococcus anginosus] >gb|EJP24466.1| putative C-S lyase [Streptococcus anginosus SK1138]	799	799	100%	0.0	99%
betaC-S lyase [Streptococcus anginosus]	798	798	100%	0.0	98%
cysteine desulfhydrase [Streptococcus anginosus group] >dbj|BAF64492.1| betaC-S lyase [Streptococcus anginosus SK52 = DSM 20563] >gb|EGL45019.	791	791	100%	0.0	97%
betaC-S lyase [Streptococcus anginosus]	788	788	100%	0.0	96%
bC-S lyase [Streptococcus constellatus]	785	785	100%	0.0	96%
cysteine desulfhydrase [Streptococcus anginosus] >gb|EFW07495.1| BC-S lyase [Streptococcus anginosus 1_2_62CV]	783	783	100%	0.0	96%
betaC-S lyase [Streptococcus anginosus C1051] >ref|WP_021001397.1| betaC-S lyase [Streptococcus anginosus] >dbj|BAF64496.1| betaC-S lyase [Strep	780	780	100%	0.0	95%
bifunctional PLP-dependent enzyme with beta-cystathionase/maltose regulon repressor activities [Streptococcus anginosus] >dbj|GAD46209.1| bifunction	778	778	100%	0.0	95%
betaC-S lyase [Streptococcus anginosus C238] >ref|WP_020999497.1| betaC-S lyase [Streptococcus anginosus] >dbj|BAN62201.1| bifunctional PLP-dep	776	776	100%	0.0	95%
betaC-S lyase [Streptococcus anginosus]	776	776	100%	0.0	95%
cysteine desulfhydrase [Streptococcus anginosus] >gb|EID23793.1| aminotransferase, class I/II [Streptococcus anginosus subsp. whileyi CCUG 39159]	774	774	100%	0.0	95%

Beyond the ten first hits

betaC-S lyase [Streptococcus constellatus]	749	749	100%	0.0	91%
betaC-S lyase [Streptococcus constellatus]	748	748	100%	0.0	91%
betaC-S lyase [Streptococcus constellatus]	747	747	100%	0.0	91%
betaC-S lyase [Streptococcus constellatus subsp. pharyngis C232] >ref|YP_008498467.1| betaC-S lyase [Streptococcus constellatus subsp. pharyngis C:	746	746	100%	0.0	91%
betaC-S lyase [Streptococcus intermedius]	746	746	100%	0.0	91%
L-cysteine desulfhydrase [Streptococcus intermedius] >gb|EKU17775.1| L-cysteine desulfhydrase [Streptococcus intermedius BA1]	744	744	100%	0.0	91%
cysteine desulfhydrase [Streptococcus intermedius] >gb|EHG12635.1| hypothetical protein HMPREF9177_00907 [Streptococcus intermedius F0413]	744	744	100%	0.0	91%
betaC-S lyase [Streptococcus constellatus]	744	744	100%	0.0	91%
betaC-S lyase [Streptococcus intermedius]	744	744	100%	0.0	91%
betaC-S lyase [Streptococcus intermedius]	743	743	100%	0.0	91%
cysteine desulfhydrase [Streptococcus intermedius] >gb|EHG13139.1| hypothetical protein HMPREF9682_01025 [Streptococcus intermedius F0395]	743	743	100%	0.0	91%
bC-S lyase [Streptococcus intermedius]	743	743	100%	0.0	91%
betaC-S lyase [Streptococcus constellatus]	742	742	100%	0.0	90%
betaC-S lyase [Streptococcus intermedius]	741	741	100%	0.0	90%
betaC-S lyase [Streptococcus intermedius C270] >ref|WP_020998948.1| betaC-S lyase [Streptococcus intermedius] >gb|AGU78427.1| betaC-S lyase [Stre	741	741	100%	0.0	90%
betaC-S lyase [Streptococcus intermedius B196] >ref|WP_021003009.1| betaC-S lyase [Streptococcus intermedius] >gb|AGU76589.1| betaC-S lyase [Stre	739	739	100%	0.0	90%
cysteine desulfhydrase [Streptococcus sp. 2_1_36FAA] >gb|EEY80157.1| hypothetical protein HMPREF0847_01575 [Streptococcus sp. 2_1_36FAA]	664	664	100%	0.0	78%
cysteine desulfhydrase [Streptococcus sp. oral taxon 056] >gb|EGP66468.1| cystathionine beta-lyase PatB [Streptococcus sp. oral taxon 056 str. F0418]	662	662	100%	0.0	77%
betaC-S lyase [Streptococcus gordonii]	657	657	100%	0.0	77%
L-cysteine desulfhydrase [Streptococcus oralis]	656	656	100%	0.0	77%
betaC-S lyase [Streptococcus gordonii]	655	655	100%	0.0	77%
betaC-S lyase [Streptococcus gordonii]	654	654	100%	0.0	77%
L-cysteine desulfhydrase [Streptococcus gordonii str. Challis substr. CH1] >ref|WP_012130693.1| cysteine desulfhydrase [Streptococcus gordonii] >gb|AB	652	652	100%	0.0	76%
cysteine desulfhydrase [Streptococcus sanguinis] >gb|EGC25620.1| aminotransferase, class I/II [Streptococcus sanguinis SK405] >gb|EGC26899.1| amir	650	650	100%	0.0	75%

Table 5. Oral bacterial species with similar enzyme activity and sequence to L-cysteine desulfhydrase produced from *streptococcus anginosus*, including oral species beyond the ten first hits.

Oral bacteria	**Identity in amino acid sequence to L-cysteine desulfhydrase in *s. anginosus* (%)**
Streptococcus constellatus	96
Streptococcus intermedius	91
Streptococcus sp. oral taxon 056	77
Streptococcus gordonii	77
Streptococcus oralis	77
Streptococcus sanguinis	75

5.3 Enzyme 2: L-methionine gamma-lyase

Query: Methionine-gamma-lyase (methione-γ-lyase)
Source (organism): *Fusobacterium nucleatum subsp. nucleatum* ATCC 25586

Gene sequence

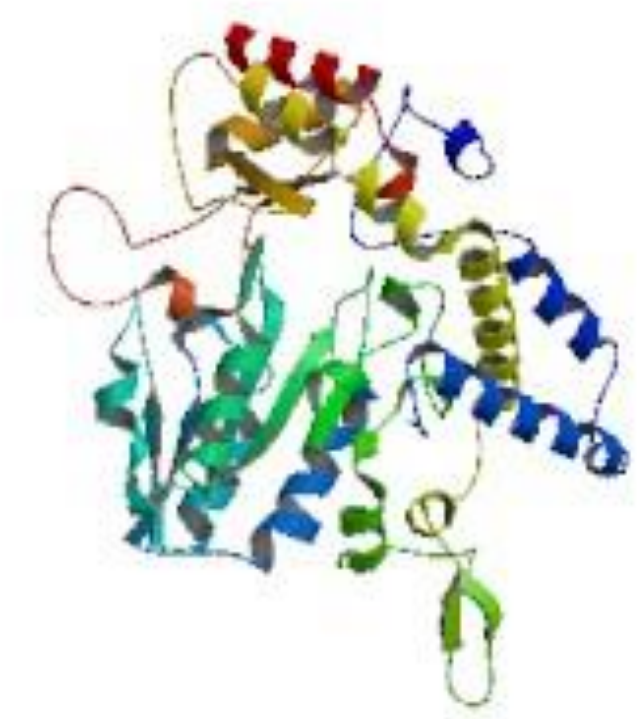
```
CTTAACATTT CGTAAAGCTG GGTTGAAATC GTGACATCTG AATTCCTTAT TAATTTCTTT
CTCATAATTC TACTCCTTCA CAGTGTACTA TGACAGTTTT TAGTATAAAT AATTTATTTA
TAAATCGTTT TATGTTAATA TTATAATATA AAAATATCAA ATATACTAGG AGGTAAATTA
TGGAAATGAA AAAATCTGGT TTAGGAACAA CTGCTATACA TGCAGGAACT TTAAAAAATT
TATATGGAAC TCTTGCAATG CCTATATATC AAACTTCTAC TTTTATATTT GATTCAGCAG
AACAAGGAGG AAGAAGATTT GCCCTTGAAG AAGCTGGATA TATTTACACA AGACTAGGCA
ATCCTACAAC AACAGTGTTA GAAAATAAAA TTGCTGCTCT TGAAGAAGGT GAAGCTGGAA
TAGCTATGTC ATCTGGTATG GGAGCTATCT CTTCAACATT GTGGACTGTA TTAAAAGCTG
GAGATCATGT TGTTACAGAT AAAACTTTAT ATGGTTGTAC TTTTGCTTTG ATGAATCATG
GACTTACAAG ATTTGGAGTT GAAGTTACTT TTGTTGATAC TTCTAATTTA GAAGAAGTTA
AAAATGCTAT GAAAAAAAAT ACAAGAGTTG TTTATCTTGA AACTCCTGCC AATCCAAATT
TAAAAATAGT TGATTTAGAA GCTTTATCTA AAATTGCTCA CACAAATCCA AATACTTTGG
TTATAGTAGA TAATACTTTT GCAACTCCAT ATATGCAAAA ACCTTTAAAA TTAGGTGTAG
ATATTGTTGT ACACTCTGCA ACTAAATATT TGAATGGACA TGGAGATGTA ATAGCAGGTC
TTGTTGTAAC AAGACAAGAA CTTGCAGATC AAATCCGTTT TGTTGGATTA AAAGATATGA
CAGGAGCTGT TTTAGGACCT CAAGAAGCAT ATTACATTAT AAGAGGATTG AAAACATTTG
AAATTCGTAT GGAAAGACAC TGTAAAAATG CAAGAACTAT TGTAGATTTC TTAAATAAAC
ATCCAAAAGT TGAAAAAGTT TATTATCCTG GACTTGAGAC TCATCCTGGT TATGAAATAG
CTAAAAAACA AATGAAAGAT TTTGGAGCAA TGATTTCATT TGAATTAAAA GGTGGCTTTG
AAGCAGGTAA AACTTTATTA AATAATTTAA AACTTTGTTC ATTAGCAGTT TCATTAGGAG
ATACTGAAAC TCTTATTCAA CACCCAGCAT CTATGACACA CTCTCCTTAT ACAAAGGAAG
AAAGAGAAGT TGCTGGAATC ACTGATGGTT TAGTTAGATT ATCAGTTGGA CTTGAAAATG
TTGAAGATAT TATAGCTGAT TTAGAACAAG GACTAGAAAA AATTTAACTT TACTCATTTG
TCTTAATTCC TTACTTGTTT AGGGTTGTTG TAAACTCATT ACAGCAACCA CTTGACAAGT
ACATAAATTA ATTCTTTAAA ATATAGGATA TGGTAAATTT TAAACTTATT AATAAAATGA
AAGAGGTAGA TATATGGAGA CTAAGGCTAG TTTTAAAGGT TTAA
```

//

Amino acid sequence

```
      1 memkksglgt taihagtlkn lygtlampiy qtstfifdsa eqggrrfale eagyiytrlg
     61 nptttvlenk iaaleegeag iamssgmgai sstlwtvlka gdhvvtdktl ygctfalmnh
    121 gltrfgvevt fvdtsnleev knamkkntrv vyletpanpn lkivdleals kiahtnpntl
    181 vivdntfatp ymqkplklgv divvhsatky lnghgdviag lvvtrqelad qirfvglkdm
    241 tgavlgpqea yyiirglktf eirmerhckn artivdflnk hpkvekvyyp glethpgyei
    301 akkqmkdfga misfelkggf eagktllnnl klcslavslg dtetliqhpa smthspytke
    361 ereaagitdg lvrlsvglen vediiadleq gleki
```

Protein structure of methionine-gamma-lyase with the same amino acid sequence (above) retrieved at MODBASE is shown in Fig.5



(Fig. 5: protein structure of methionine-gamma-lyase, http://modbase.compbio.ucsf.edu/modbasecgi/model_details.cgi?queryfile=1379270757_6340&searchmode=default&displaymode=moddetail&referer=yes&snpflag=&)

Blast result

Top first ten bacteria with similar amino acid sequence to methionine-γ-lyase found in *Fusobacterium nucleatum subsp. nucleatum*

Sequences producing significant alignments:

Select: All None Selected:0

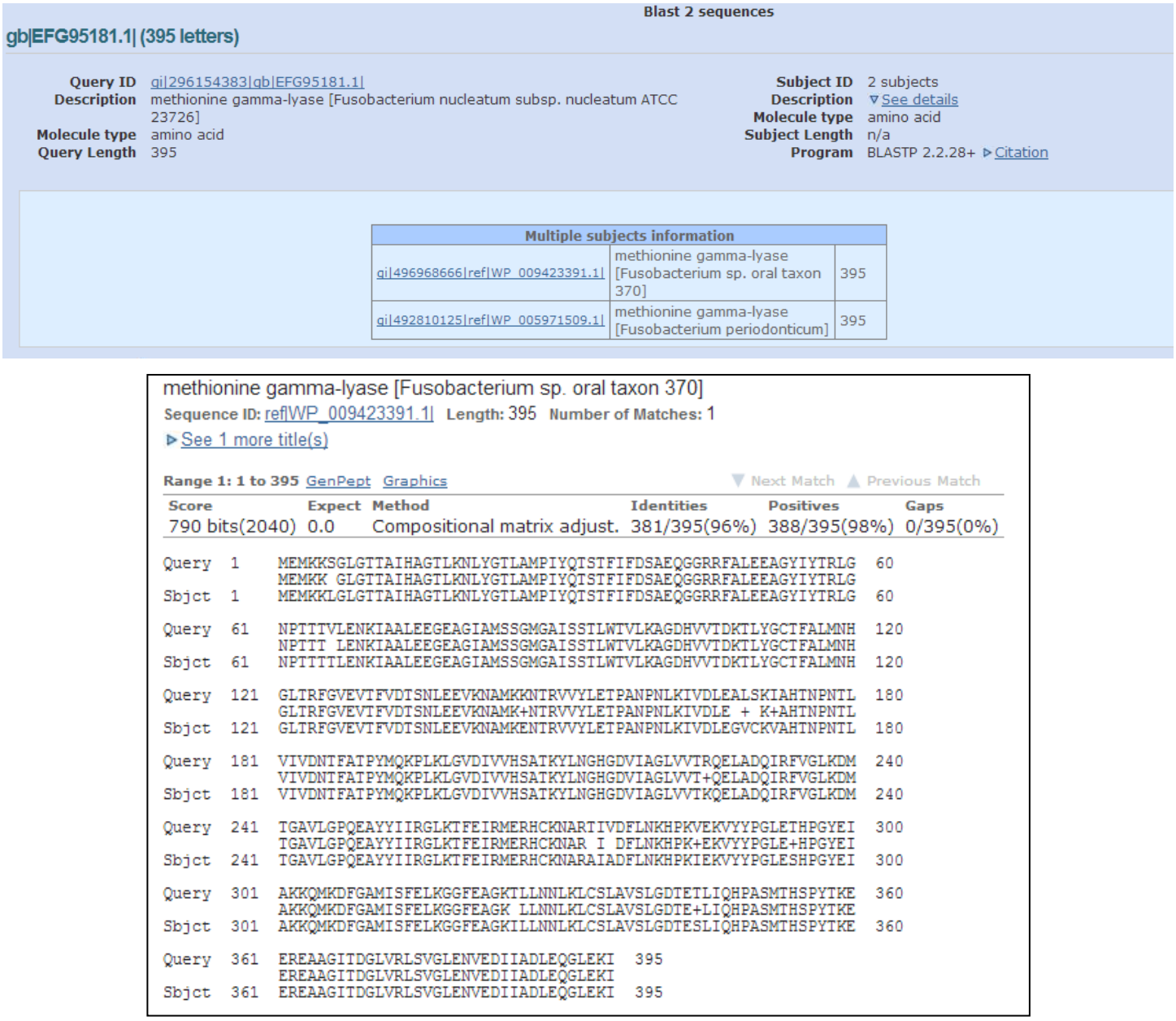
Alignments Download GenPept Graphics Distance tree of results Multiple alignment

Description	Max score	Total score	Query cover	E value	Ident
methionine gamma-lyase [Fusobacterium nucleatum] >gb|EFG95181.1| methionine gamma-lyase [Fusobacterium nucleatum subsp. nucleatum ATCC	812	812	100%	0.0	100%
methionine gamma-lyase [Fusobacterium nucleatum subsp. nucleatum ATCC 25586] >ref|WP_011017138.1| methionine gamma-lyase [Fusobacterium	811	811	100%	0.0	99%
methionine gamma-lyase [Fusobacterium sp. oral taxon 370] >gb|EHI79026.1| hypothetical protein HMPREF9093_00731 [Fusobacterium sp. oral taxon	790	790	100%	0.0	96%
methionine gamma-lyase [Fusobacterium nucleatum subsp. animalis 4_8] >ref|WP_005910894.1| methionine gamma-lyase [Fusobacterium nucleatum	789	789	100%	0.0	96%
methionine gamma-lyase [Fusobacterium sp. CAG:649]	788	788	100%	0.0	96%
methionine gamma-lyase [Fusobacterium nucleatum] >gb|EGN65482.1| methionine gamma-lyase [Fusobacterium nucleatum subsp. animalis 21_1A]	788	788	100%	0.0	96%
methionine gamma-lyase [Fusobacterium nucleatum subsp. vincentii 3_1_36A2] >ref|WP_008797734.1| methionine gamma-lyase [Fusobacterium nuc	784	784	100%	0.0	95%
methionine gamma-lyase [Fusobacterium nucleatum] >dbj|BAC02724.1| L-methionine-alpha-deamino-gamma- mercaptomethane-lyase [Fusobacteriu	774	774	100%	0.0	94%
methionine gamma-lyase [Fusobacterium nucleatum] >gb|EJU06746.1| methionine gamma-lyase [Fusobacterium nucleatum ChDC F128]	769	769	100%	0.0	93%
methionine gamma-lyase [Fusobacterium periodonticum] >gb|EFE87423.1| methionine gamma-lyase [Fusobacterium periodonticum ATCC 33693]	766	766	100%	0.0	92%
methionine gamma-lyase [Fusobacterium periodonticum] >gb|EEO38507.1| methionine gamma-lyase [Fusobacterium periodonticum 2_1_31] >gb|EKA	765	765	100%	0.0	92%

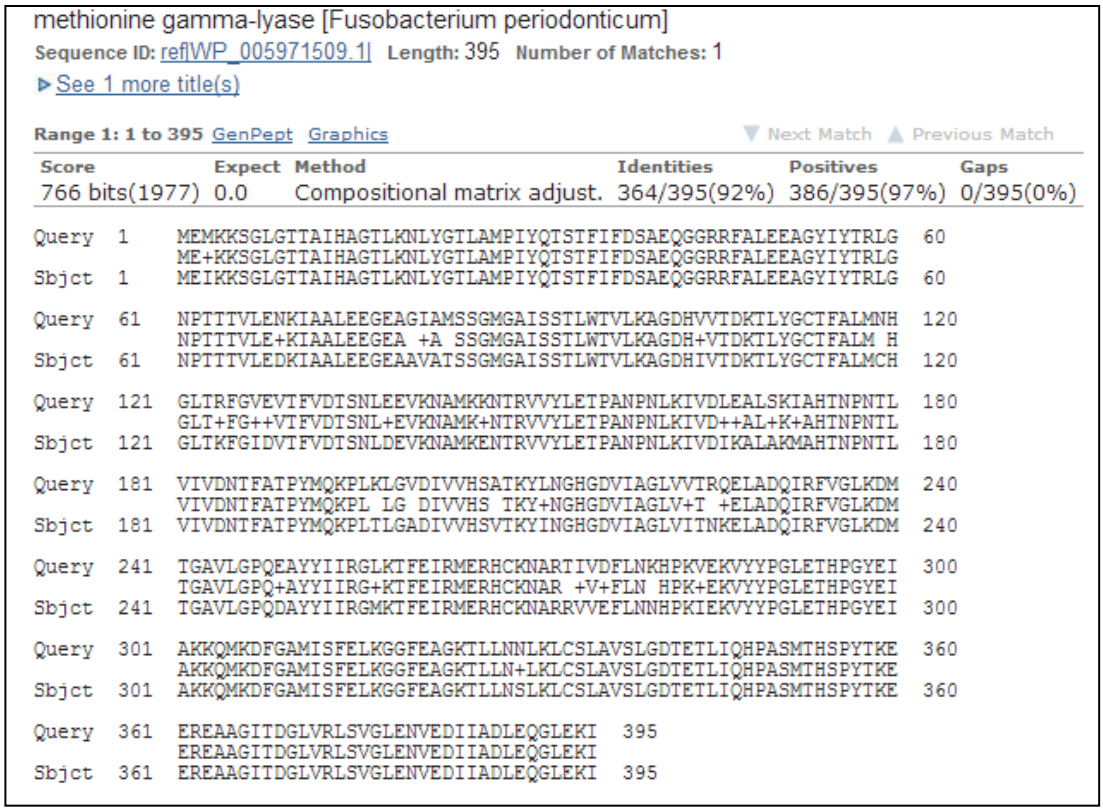
Table 6. Oral bacterial species with similar enzyme activity and sequence to methione-γ-lyase in f. nucleatum subsp. nucleatum from first ten hits.

Oral bacteria	**Identity in amino acid sequence to methione-γ-lyase in f. nucleatum subsp. nucleatum (%)**
Fusobacterium sp. oral taxon 370	96
Fusobacterium periodonticum	92

Delta Blast result



(Fig. 6: showing the two comparing subjects; query = methionine-γ-lyase in *Fusobacterium nucleatum subsp. nucleatum* and subject 1= methionine-γ-lyase in *f.sp.oral taxon 370*)


methionine gamma-lyase [Fusobacterium periodonticum]

Sequence ID: ref|WP_005971509.1| Length: 395 Number of Matches: 1

See 1 more title(s)

Range 1: 1 to 395 GenPept Graphics Next Match Previous Match

Score	Expect	Method	Identities	Positives	Gaps
766 bits(1977)	0.0	Compositional matrix adjust.	364/395(92%)	386/395(97%)	0/395(0%)

```
Query  1    MEMKKSGLGTTAIHAGTLKNLYGTLAMPIYQTSTFIFDSAEQGGRRFALEEAGYIYTRLG  60
            ME+KKSGLGTTAIHAGTLKNLYGTLAMPIYQTSTFIFDSAEQGGRRFALEEAGYIYTRLG
Sbjct  1    MEIKKSGLGTTAIHAGTLKNLYGTLAMPIYQTSTFIFDSAEQGGRRFALEEAGYIYTRLG  60

Query  61   NPTTTVLENKIAALEEGEAGIAMSSGMGAISSTLWTVLKAGDHVVTDKTLYGCTFALMNH  120
            NPTTTVLE+KIAALEEGEA +A SSGMGAISSTLWTVLKAGDH+VTDKTLYGCTFALM H
Sbjct  61   NPTTTVLEDKIAALEEGEAAVATSSGMGAISSTLWTVLKAGDHIVTDKTLYGCTFALMCH  120

Query  121  GLIRFGVEVTFVDTSNLEEVKNAMKKNTRVVYLETPANPNLKIVDLEALSKIAHTNPNTL  180
            GLT+FG++VTFVDTSNL+EVKNAMK+NTRVVYLETPANPNLKIVD++AL+K+AHTNPNTL
Sbjct  121  GLTKFGIDVTFVDTSNLDEVKNAMKENTRVVYLETPANPNLKIVDIKALAKMAHTNPNTL  180

Query  181  VIVDNTFATPYMQKPLKLGVDIVVHSATKYLNGHGDVIAGLVVTRQELADQIRFVGLKDM  240
            VIVDNTFATPYMQKPL LG DIVVHS TKY+NGHGDVIAGLV+T +ELADQIRFVGLKDM
Sbjct  181  VIVDNTFATPYMQKPLTLGADIVVHSVTKYINGHGDVIAGLVITNKELADQIRFVGLKDM  240

Query  241  TGAVLGPQEAYYIIRGLKTFEIRMERHCKNARTIVDFLNKHPKVEKVYYPGLETHPGYEI  300
            TGAVLGPQ+AYYIIRG+KTFEIRMERHCKNAR +V+FLN HPK+EKVYYPGLETHPGYEI
Sbjct  241  TGAVLGPQDAYYIIRGMKTFEIRMERHCKNARRVVEFLNNHPKIEKVYYPGLETHPGYEI  300

Query  301  AKKQMKDFGAMISFELKGGFEAGKTLLNNLKLCSLAVSLGDTETLIQHPASMTHSPYTKE  360
            AKKQMKDFGAMISFELKGGFEAGKTLLN+LKLCSLAVSLGDTETLIQHPASMTHSPYTKE
Sbjct  301  AKKQMKDFGAMISFELKGGFEAGKTLLNSLKLCSLAVSLGDTETLIQHPASMTHSPYTKE  360

Query  361  EREAAGITDGLVRLSVGLENVEDIIADLEQGLEKI  395
            EREAAGITDGLVRLSVGLENVEDIIADLEQGLEKI
Sbjct  361  EREAAGITDGLVRLSVGLENVEDIIADLEQGLEKI  395
```

(Fig. 7: showing the two comparing subjects; query = methionine-γ-lyase in *Fusobacterium nucleatum subsp. nucleatum* and subject 1= methionine-γ-lyase in *f.periodonticum*)

Another organism that produces methionine gamma-lyase is *Treponema denticola*, the amino acid sequence are analyzed using BLAST.

Query: Methionine gamma-lyase
Source (organism): *Treponema denticola* ATCC 35405

Amino acid sequence

```
  1 mnrkeleklg faskqihags iknkygalat piyqtstfaf dsaeqggrrf aleeegyiyt
 61 rlgnptttvv eeklacleng eacmsassgi gavtsciwsi vnagdhivag ktlygctfaf
121 lnhglsrfgv dvtfvdtrdp envkkalkpn tkivyletpa npnmylcdia avskiahahn
181 peckvivdnt ymtpylqrpl dlgadvvlhs atkylnghgd viagfvvgkk efidqvrfvg
241 vkdmtgstlg pfeayligrg mktldirmek hcanaqkvae flekhpaves iafpglksfp
301 qyelakkqmk lcgamiaftv kggleagktl insvkfatia vslgdaetli qhpasmthsp
361 ytpeeraasd iaeglvrlsv gledaediia dlkqaldklv k
```

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics Distance tree of results Multiple alignment

Description	Max score	Total score	Query cover	E value	Ident
methionine gamma-lyase [Treponema denticola ATCC 35405] >ref|WP_002674490.1| methionine gamma-lyase [Treponema denticola] >gb|AAS12720.	830	830	100%	0.0	100%
methionine gamma-lyase [Treponema denticola] >gb|EMB30126.1| methionine gamma-lyase [Treponema denticola MYR-T] >gb|EMB31281.1| methioni	829	829	100%	0.0	99%
methionine gamma-lyase [Treponema denticola] >gb|EMB26972.1| methionine gamma-lyase [Treponema denticola SP37] >gb|EPF33501.1| methionin	829	829	100%	0.0	99%
methionine gamma-lyase [Treponema denticola] >gb|EMB47709.1| methionine gamma-lyase [Treponema denticola ASLM] >gb|EMD55891.1| methionir	828	828	100%	0.0	99%
methionine gamma-lyase [Treponema denticola] >gb|EMB24503.1| methionine gamma-lyase [Treponema denticola SP33] >gb|EPF37847.1| methionin	828	828	100%	0.0	99%
methionine gamma-lyase [Treponema denticola] >gb|EGC78268.1| methionine gamma-lyase [Treponema denticola F0402]	826	826	100%	0.0	99%
methionine gamma-lyase [Treponema pedis str. T A4] >ref|WP_020965710.1| methionine gamma-lyase [Treponema pedis] >gb|AGT44412.1| methionir	738	738	99%	0.0	87%
methionine gamma-lyase [Treponema phagedenis] >gb|EFW39355.1| methionine gamma-lyase [Treponema phagedenis F0421]	690	690	99%	0.0	82%
methionine gamma-lyase [Porphyromonas cansulci JCM 13913]	641	641	99%	0.0	75%
methionine gamma-lyase [Porphyromonas uenonis] >gb|EEK17527.1| methionine gamma-lyase [Porphyromonas uenonis 60-3]	640	640	99%	0.0	75%
methionine gamma-lyase [Porphyromonas endodontalis] >gb|EEN83521.1| methionine gamma-lyase [Porphyromonas endodontalis ATCC 35406]	640	640	99%	0.0	75%
methionine gamma-lyase [Porphyromonas crevioricanis JCM 15906]	639	639	99%	0.0	74%
methionine gamma-lyase [Porphyromonas asaccharolytica DSM 20707] >ref|WP_004330655.1| methionine gamma-lyase [Porphyromonas asaccharol	639	639	99%	0.0	74%
methionine gamma-lyase [Porphyromonas macacae]	629	629	98%	0.0	75%
methionine gamma-lyase [Peptoniphilus sp. oral taxon 386] >gb|EFI41605.1| methionine gamma-lyase [Peptoniphilus sp. oral taxon 386 str. F0131]	626	626	99%	0.0	73%
methionine gamma-lyase [Porphyromonas gingivalis] >gb|EOA11211.1| methionine gamma-lyase [Porphyromonas gingivalis JCVI SC001] >gb|ERJ656	625	625	99%	0.0	75%

Table 7. Oral bacterial species identified from Blast result of methionine-γ-lyase in *treponema denticola*

Oral bacteria	**Identity in amino acid sequence to methionine-γ-lyase in** *treponema denticola* **(%)**
Porphyromonas endotalis (ATCC 35406)	75
Porphyromonas gingivalis	75

5.4 **Enzyme 3: L-methionine-alpha-deamino-gamma-mercaptomethane-lyase**

Query: L-methionine-alpha-deamino-gamma-mercaptomethane-lyase (MET-ase)
Source/ organism: *Fusobacterium nucleatum subsp. polymorphum*¸ATCC 10953

Blast result: Bacteria containing similar enzyme to MET-ase found in *Fusobacterium nucleatum subsp. polymorphum*¸ATCC 10953 are:

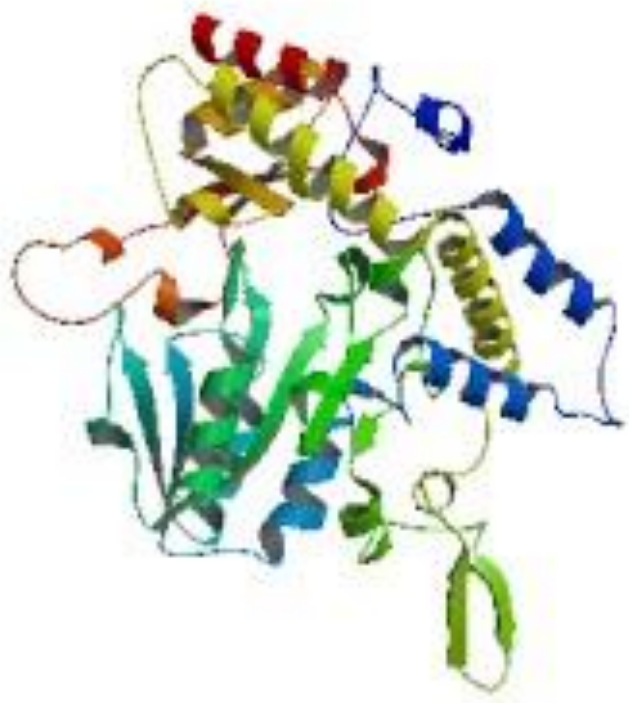
Gene sequence

```
        1 tttataaatc gttttatgtt aatattataa tataaaaaca tcaaatatac taggaggtaa
       61 attatggaaa cgaaaaaata tggtttagga acaactgcta tacatgcagg aactttaaaa
      121 aatttatatg gaactcttgc aatgccaata tatcaaactt ctacttttat atttgactca
      181 gctgaacaag gtggaagaag atttgctctt gaagaagctg gatatattta tacaagatta
      241 gggaatccta caacaacagt tttagaaaat aaaattgcag ctcttgaaga aggagaagct
      301 gctgttgcta catcatctgg tatgggagct atatcttcaa cattatggac tgttttaaaa
      361 gcaggggatc atgttgttac tgataaaact ttatatggtt gtacttttgc tttaatgtgt
      421 catggactta caagatttgg aatagaagtt acttttgttg atacttcaaa tttagatgaa
      481 gttaaaaatg ctatgaaaaa aaatacaaga gttgtttatc ttgaaacacc tgctaaccca
      541 aatttaaaaa tagttgattt agaagcactt tctaaacttg ctcatacaaa tccaaatact
      601 ttggttattg ttgacaatac ttttgcaact ccatatatgc aaaaaccttt aaaattaggt
      661 gcagatattg ttgttcactc tgtaacaaaa tatataaacg gacatggaga tgtaatagca
      721 ggtcttgtta taacaaataa agaacttgca gatcaaattc gttttatagg tctaaaagat
      781 atgacaggag cagttttagg accacaagat gcttattata tcattagagg tatgaaaact
      841 tttgaaattc gtatggaaag acattgtaaa aatgctaaaa aagttgttga atttttaaat
      901 aaacacccaa aaattgaaag agtttattat cctggacttg aaacacaccc tggtcatgaa
      961 atagcaaaaa aacaaatgaa agattttgga gcaatgattt cttttgaact aaaaggtggt
     1021 tttgaagcag gtaaaacttt actaaataac ttaaaacttt gttcattagc tgtttcattg
     1081 ggagatactg aaactcttat tcaacaccca gcatctatga cacactcacc ttatacaaaa
     1141 gaagaaagag aagctgctgg aataactgat ggcttggtta gattatcagt tggtcttgaa
     1201 aatgttgaag atattatagc agatttggaa caaggactag aaaaaattta attttactca
     1261 tttatcttca ttccttactt gtttatggtt gttgnaatag agttttacca acaacccatt
     1321 taaccaaac
//
```

Amino acid sequence

```
        1 metkkyglgt taihagtlkn lygtlampiy qtstfifdsa eqggrrfale eagyiytrlg
       61 nptttvlenk iaaleegeaa vatssgmgai sstlwtvlka gdhvvtdktl ygctfalmch
      121 gltrfgievt fvdtsnldev knamkkntrv vyletpanpn lkivdleals klahtnpntl
      181 vivdntfatp ymqkplklga divvhsvtky inghgdviag lvitnkelad qirfiglkdm
      241 tgavlgpqda yyiirgmktf eirmerhckn akkvveflnk hpkiervyyp glethpghei
      301 akkqmkdfga misfelkggf eagktllnnl klcslavslg dtetliqhpa smthspytke
      361 ereaagitdg lvrlsvglen vediiadleq gleki
```

Protein structure of L-methionine-alpha-deamino-gamma-mercaptomethane-lyase with the same amino acid sequence as above retrieved at MODBASE.



(Fig. 8: Protein structure of MET-ase, http://modbase.compbio.ucsf.edu/modbasecgi/model_details.cgi?queryfile=1379270398_9395&searchmode=default&displaymode=moddetail&referer=yes&snpflag=&)

Blast result

Top first ten bacteria with similar amino acid sequence to MET-ase found in *Fusobacterium nucleatum subsp. polymorphum*

Sequences producing significant alignments:

Select: All None Selected:0

Alignments Download GenPept Graphics Distance tree of results Multiple alignment

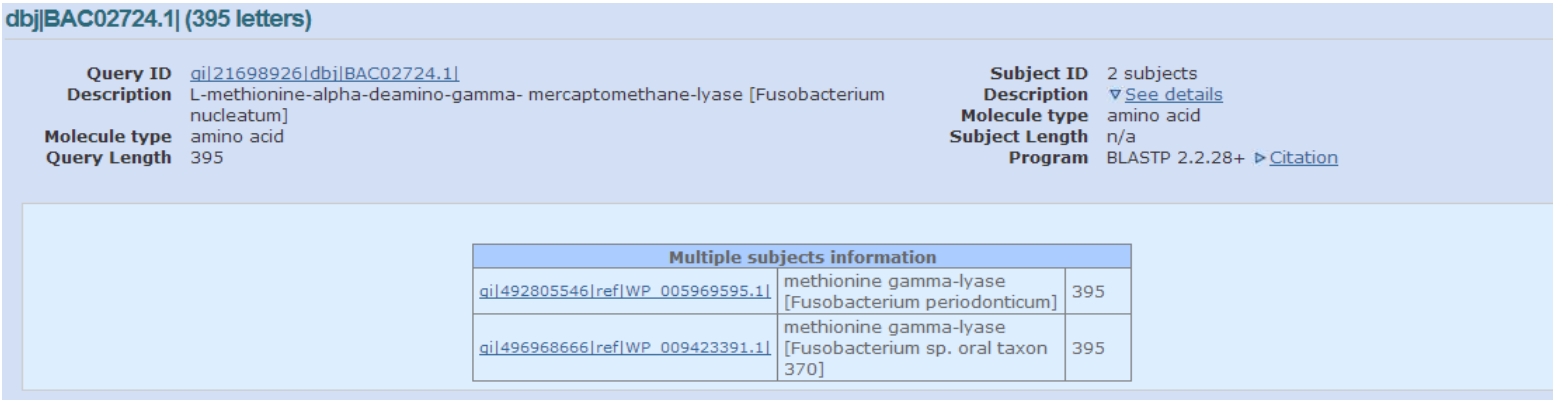
Description	Max score	Total score	Query cover	E value	Ident
methionine gamma-lyase [Fusobacterium nucleatum] >dbj|BAC02724.1| L-methionine-alpha-deamino-gamma- mercaptomethane-lyase [Fusobacteriu	812	812	100%	0.0	100%
methionine gamma-lyase [Fusobacterium nucleatum] >gb|EJU06746.1| methionine gamma-lyase [Fusobacterium nucleatum ChDC F128]	802	802	100%	0.0	99%
methionine gamma-lyase [Fusobacterium periodonticum] >gb|EEO38507.1| methionine gamma-lyase [Fusobacterium periodonticum 2_1_31] >gb|EKA	788	788	100%	0.0	96%
methionine gamma-lyase [Fusobacterium nucleatum subsp. vincentii 3_1_36A2] >ref|WP_008797734.1| methionine gamma-lyase [Fusobacterium nuc	786	786	100%	0.0	96%
methionine gamma-lyase [Fusobacterium periodonticum] >gb|EFG27715.1| methionine gamma-lyase [Fusobacterium periodonticum 1_1_41FAA]	786	786	100%	0.0	96%
methionine gamma-lyase [Fusobacterium periodonticum] >gb|EFE87423.1| methionine gamma-lyase [Fusobacterium periodonticum ATCC 33693]	784	784	100%	0.0	95%
methionine gamma-lyase [Fusobacterium nucleatum] >gb|EFG95181.1| methionine gamma-lyase [Fusobacterium nucleatum subsp. nucleatum ATCC	774	774	100%	0.0	94%
methionine gamma-lyase [Fusobacterium nucleatum subsp. nucleatum ATCC 25586] >ref|WP_011017138.1| methionine gamma-lyase [Fusobacteriun	772	772	100%	0.0	93%
methionine gamma-lyase [Fusobacterium nucleatum subsp. animalis 4_8] >ref|WP_005910894.1| methionine gamma-lyase [Fusobacterium nucleatur	764	764	100%	0.0	92%
methionine gamma-lyase [Fusobacterium sp. CAG:649]	763	763	100%	0.0	92%
methionine gamma-lyase [Fusobacterium nucleatum] >gb|EGN65482.1| methionine gamma-lyase [Fusobacterium nucleatum subsp. animalis 21_1A]	763	763	100%	0.0	92%

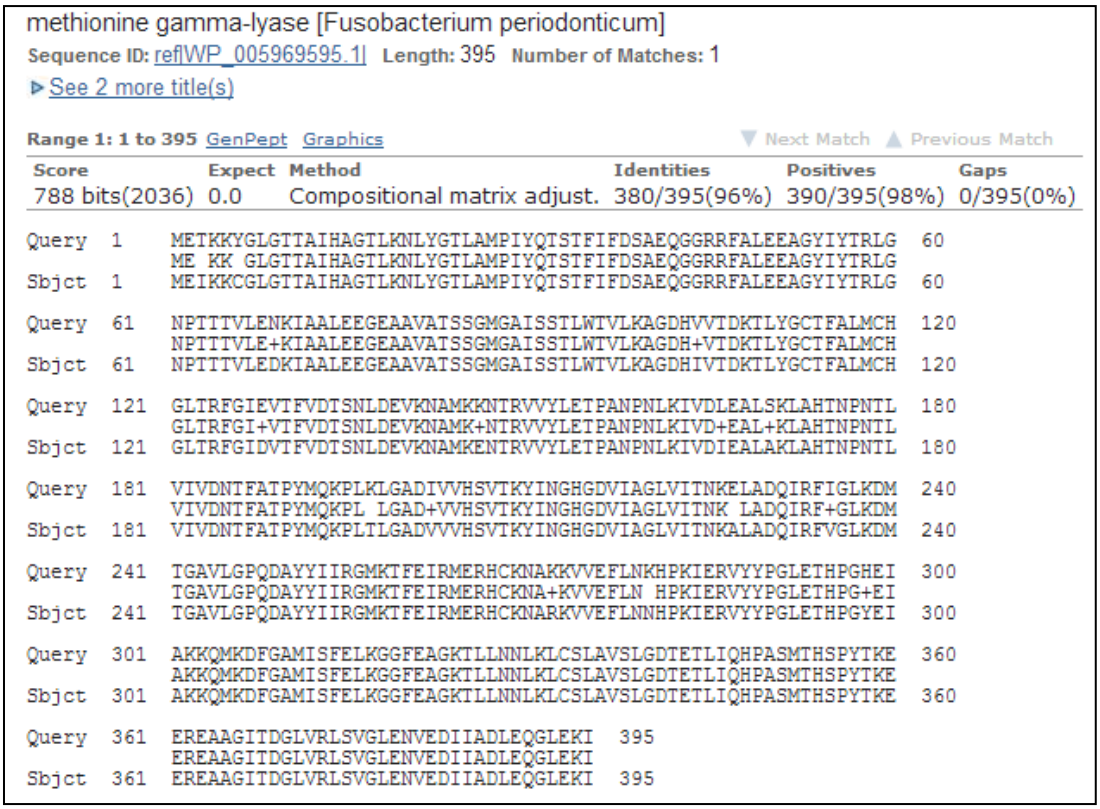
Oral bacteria identified on the first ten hits and number 11 on the list (not shown above), which contains similar enzyme to MET-ase found in *Fusobacterium nucleatum subsp. polymorphum* are:

Table 8. Oral bacterial species identified from the first ten hits, including number 11 from Blast result of MET-ase in *Fusobacterium nucleatum subsp. polymorphum*¸ATCC 10953

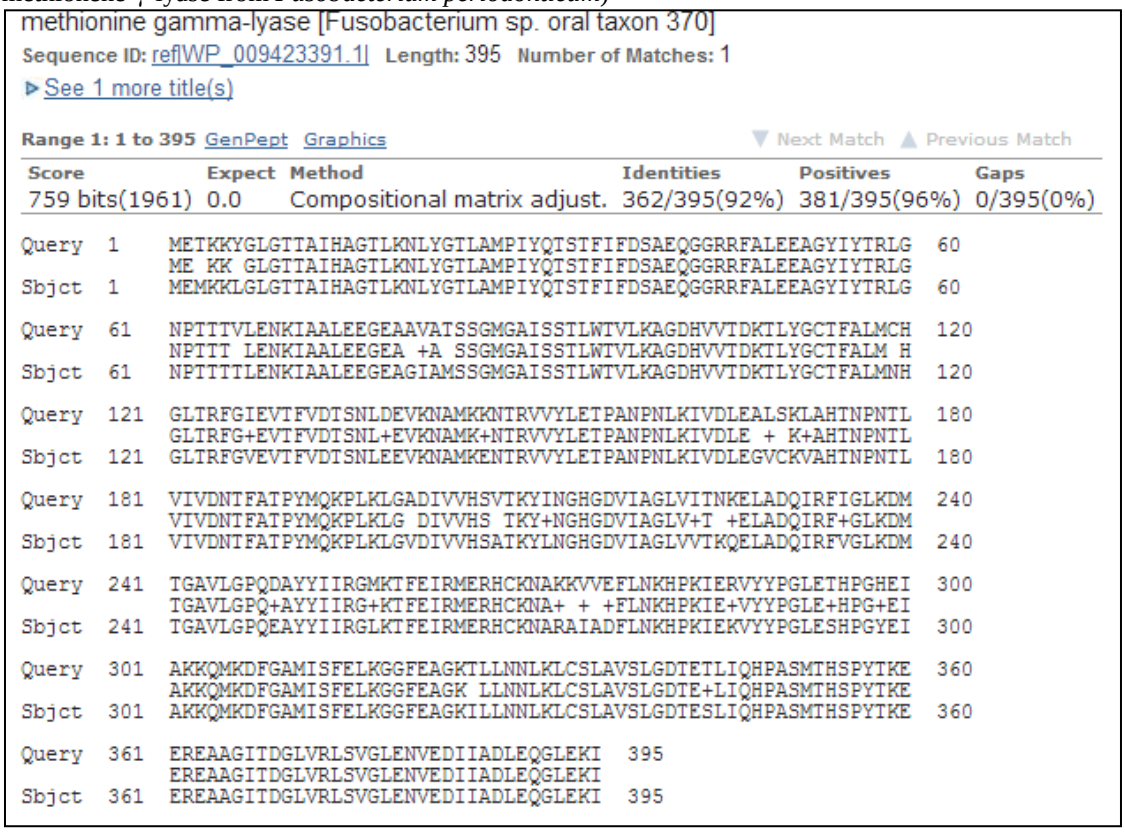
Oral bacteria	**Identity in amino acid sequence to MET-ase in *Fusobacterium nucleatum subsp. polymorphum*¸ATCC 10953 (%)**
Fusobacterium periodonticum	96
Fusobacterium sp. oral taxon 370	92

Delta Blast result





(Fig. 9: query = MET-ase from *Fusobacterium nucleatum subsp. polymorphum*¸ ATCC 10953 and subject 1= methionene-γ-lyase from *Fusobacterium periodonticum*)



methionine gamma-lyase [Fusobacterium sp. oral taxon 370]

Sequence ID: ref|WP_009423391.1| Length: 395 Number of Matches: 1

▶ See 1 more title(s)

Range 1: 1 to 395 GenPept Graphics ▼ Next Match ▲ Previous Match

Score	Expect	Method	Identities	Positives	Gaps
759 bits(1961)	0.0	Compositional matrix adjust.	362/395(92%)	381/395(96%)	0/395(0%)

```
Query  1    METKKYGLGTTAIHAGTLKNLYGTLAMPIYQTSTFIFDSAEQGGRRFALEEAGYIYTRLG  60
            ME KK GLGTTAIHAGTLKNLYGTLAMPIYQTSTFIFDSAEQGGRRFALEEAGYIYTRLG
Sbjct  1    MEMKKLGLGTTAIHAGTLKNLYGTLAMPIYQTSTFIFDSAEQGGRRFALEEAGYIYTRLG  60

Query  61   NPTTTVLENKIAALEEGEAAVATSSGMGAISSTLWTVLKAGDHVVTDKTLYGCTFALMCH  120
            NPTTT LENKIAALEEGEA +A SSGMGAISSTLWTVLKAGDHVVTDKTLYGCTFALM H
Sbjct  61   NPTTTTLENKIAALEEGEAGIAMSSGMGAISSTLWTVLKAGDHVVTDKTLYGCTFALMNH  120

Query  121  GLTRFGIEVTFVDTSNLDEVKNAMKKNTRVVYLETPANPNLKIVDLEALSKLAHTNPNTL  180
            GLTRFG+EVTFVDTSNL+EVKNAMK+NTRVVYLETPANPNLKIVDLE + K+AHTNPNTL
Sbjct  121  GLTRFGVEVTFVDTSNLEEVKNAMKENTRVVYLETPANPNLKIVDLEGVCKVAHTNPNTL  180

Query  181  VIVDNTFATPYMQKPLKLGADIVVHSVTKYINGHGDVIAGLVITNKELADQIRFIGLKDM  240
            VIVDNTFATPYMQKPLKLG DIVVHS TKY+NGHGDVIAGLV+T +ELADQIRF+GLKDM
Sbjct  181  VIVDNTFATPYMQKPLKLGVDIVVHSATKYLNGHGDVIAGLVVIKQELADQIRFVGLKDM  240

Query  241  TGAVLGPQDAYYIIRGMKTFEIRMERHCKNAKKVVEFLNKHPKIERVYYPGLETHPGHEI  300
            TGAVLGPQ+AYYIIRG+KTFEIRMERHCKNA+ + +FLNKHPKIE+VYYPGLE+HPG+EI
Sbjct  241  TGAVLGPQEAYYIIRGLKTFEIRMERHCKNARAIADFLNKHPKIEKVYYPGLESHPGYEI  300

Query  301  AKKQMKDFGAMISFELKGGFEAGKTLLNNLKLCSLAVSLGDTETLIQHPASMTHSPYTKE  360
            AKKQMKDFGAMISFELKGGFEAGK LLNNLKLCSLAVSLGDTE+LIQHPASMTHSPYTKE
Sbjct  301  AKKQMKDFGAMISFELKGGFEAGKILLNNLKLCSLAVSLGDTESLIQHPASMTHSPYTKE  360

Query  361  EREAAGITDGLVRLSVGLENVEDIIADLEQGLEKI  395
            EREAAGITDGLVRLSVGLENVEDIIADLEQGLEKI
Sbjct  361  EREAAGITDGLVRLSVGLENVEDIIADLEQGLEKI  395
```

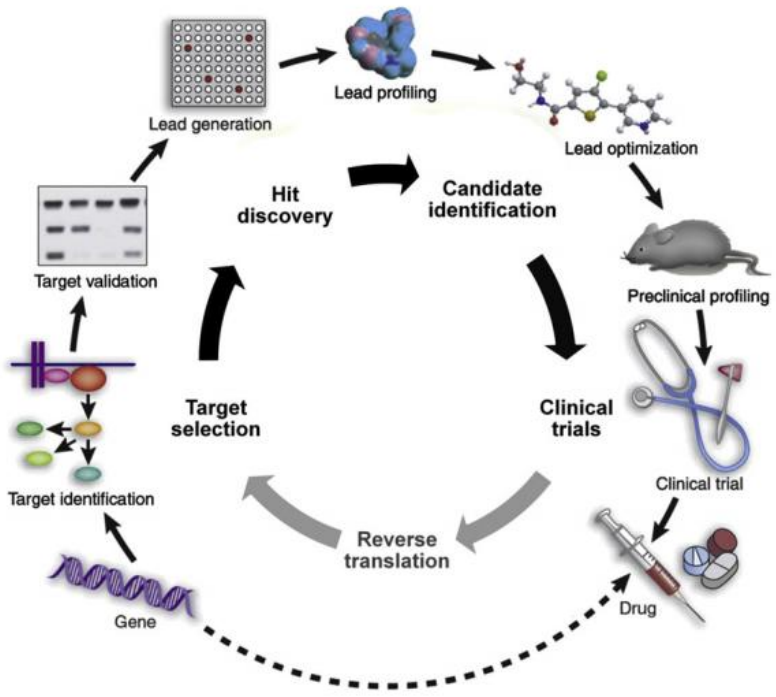
(Fig. 10: Query 1= MET-ase in *Fusobacterium nucleatum subsp. polymorphum*¸ ATCC 10953, subject 1 = methionene-γ-lyase in *Fusobacterium sp. oral taxon 370*)

6. Future Prospective in Controlling Halitosis

Since halitosis is caused primarily by releasing sulfur compounds (H_2S and methyl mercaptan (See 4.1) and these reactions are catalyzed by enzymes expressed in specific oral bacteria containing genes encoding these enzymes (section 3&5) steps should be taken to develop specific, effective anti-halitosis product that are not currently available.

The following is a brief summary of steps that should be adopted in the discovery of potential anti-halitosis drugs. These outlines steps are currently the main steps followed in drug discovery in general (17). The basic outline for drug discovery can be divided into five main steps, illustrated below.

6.1 Target selection and validation
6.2 Chemical hit and lead generation
6.3 Lead optimization to select a clinical candidate (two different methods to select leads).
6.4 Preclinical studies
6.5 Clinical trials
(17)



(Fig. 11: steps involved in drug discovery and development: from gene to drug) (17)

The period of research until the registration of a new drug may take 10-15 years. This is the pathway that ultimately leads to the choice of a new chemical entity, a drug substance, having properties which can be administered to humans in clinical trials, and then can be approved for marketing, having as main characteristics clinical efficacy and clinical safety (28).

6.1 Target selection and validation

The way in selecting a specific target in the elimination of halitosis is to inhibit the enzymes that catalyzes the reaction of volatile sulfur compounds. Selecting the right target is a question of balancing opportunities with risks, taking into account two important questions in assessing the overall risk prior to moving to step two is crucial:

- will inhibiting the target show desired biological and therapeutic effect in patient (biological risk)?

- is it possible to discover an inhibitor that acts on a target and exhibit drug-like properties be discovered (feasibility risk) (17).

One way in selecting a target for halitosis drug discovery is homology modeling, which is one of the first steps in virtual screening (in-silico screening, Table 10) a method based on the assumptions that proteins that possess similar sequences share similar three-dimensional structures, and only a limited number of protein folds exist in nature. Homology modeling has been stated as the best structure prediction method of homologous protein so far, and it was widely used in structure-based drug discovery projects (26).

In discovering anti-halitosis drug, the main candidate targets would be; L-cysteine desulfhydrase, methionine gamma-lyase and L-methionine-alpha-deamino-gamma-mercaptomethane-lyase (MET-ase). A theory we conclude from previous studies on halitosis and the enzymatic reactions involved.

"Inhibiting the main enzymes catalyzing production reaction will show no reduction in the sulfur production in halitosis".

This is only a hypothesis, a hypothesis we need to design an assay to validate the inhibitor candidates of choice.

Assessment of the validity of the given targets

Having established the targets of interest, the second step is assessing the validity of the targets, which is to ensure and to increase our confidence in the hypothesis that inhibiting these targets will lead to desired therapeutic effects in patients.

First we need a physical amount of the target, using the known gene sequence, the gene is cloned and the production of the target enzyme can be done using recombinant DNA technology. Second, an assessment of the target is done through enzymatic assay, visualizing the enzyme when it is present. A brief example on an enzymatic assay to visualize hydrogen sulfide (product) and MET-ase (enzyme) are explained in a study published by Fukamachi et Al "High production of methyl mercaptan by L-methionine-a-deamino-c-mercaptomethane lyase from *Treponema denticola*". Here the author mentioned bismuth together with hydrogen sulfide produces a black precipitate.

Hydrogen sulfide produced by oral bacteria reacts with bismuth chloride to form bismuth sulfide as a black precipitate, as described by the following reaction (18):

$$3H_2S + 2BiCl_3 \rightarrow Bi_2S_3\downarrow + 6HCl \quad (18)$$

Hydrogen sulfide–producing bacteria can be detected by measuring the absorbance of the black precipitate.

In evaluating enzymatic activity of MET-ase, Fukamachi (12) purified MET-ase using expression vector containing megL-gene, which is the gene coding for MET-ase in *T.denticola*. Using a sodium dodecyl sulfate polyacrylamide gel electrophoresis technique (SDS-page gel) one can visualize the amount of MET-ase present. Further the enzyme travels suggest a small amount MET-ase being produced (12).

6.2 Chemical hit and lead compounds generation

Two overall types of approaches can be distinguished (Table 10):

A) Random screening (High throughput screening)
B) Virtual screening (In-silico screening)
(17)

Table 10. Two methods of drug-screening; Random (High-throughput) screening and In-silico screening (30).

Details	**Random screening (high-throughput screening)**	**Virtual screening (in-silico screening)**
Requirements	Development of an assay to inhibit the activity of the enzyme(s) using non-toxic chemical libraries	Resolving the 3D-structure and modeling of the enzymes catalyzing the VSC if it is not known
Compound library	Pre-synthesize compounds, usually from corporate inventories	Compounds in e-format
Tools/ hardware	Micro titer plates, plate controls, reagents, readout devices and analysis software	Structure- or ligand-based screening software; computing resources
Evaluation of hits	Statistical comparison where active agents (‘hits’) lie outside the mean response for all tested agents by some predetermined factor based on the organization’s threshold for cost and test capacity	Scoring and ranking; visual inspection to detect presence of key interactions, chemical clustering

A-Random screening

This requires no previous knowledge of target structure or of the inhibitor. This method involves randomly screening of some thousand compounds that are already known, most from plant extract. Each and every compound is tested, putting them into test tubes with oral bacterial broths to see which tube will give a positive hit.

B-Virtual screening (in-silico screening)

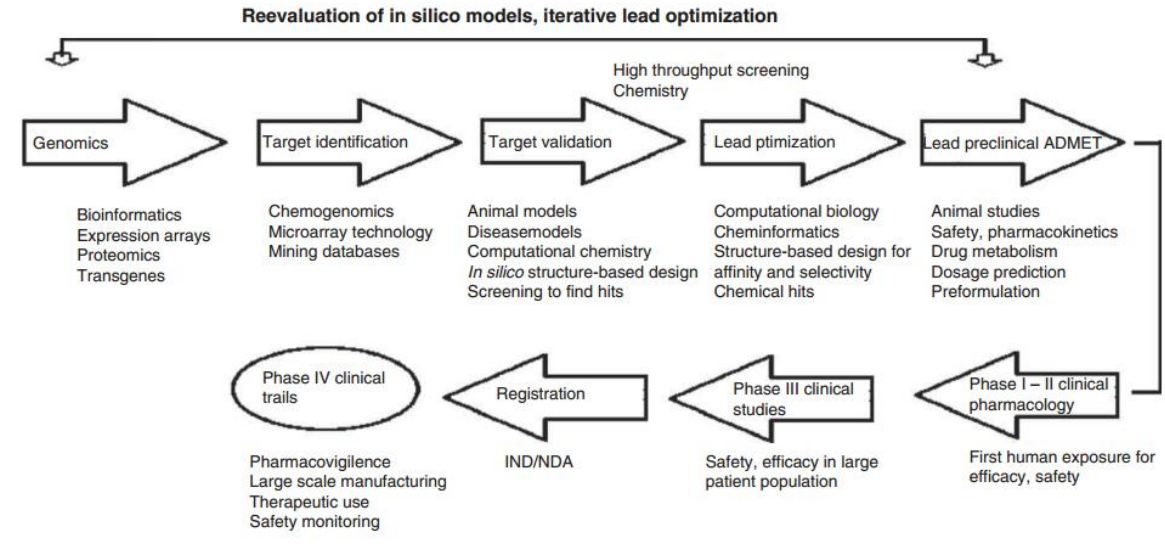
The approach of selecting compounds from large databases by using computational tools rather than physically screening them is generally referred to as virtual screening. Conceptually two different approaches can be followed:

- Ligand-based approaches select compounds from databases that are in one way or another similar to an already existing inhibitor of the target in question (Schneider, 2010).

- Structure-based approaches seek to evaluate computationally the fit of compounds to a binding pocket.

The compounds are then ranked by the predicted affinity and only the top 100-1000 compounds are screened. Virtual screening has obvious advantages over physical screening. It is significantly less resource-intensive and faster. In addition, even compounds that are not available can be evaluated by virtual screening and if found promising, can be bought or synthesized. Millions of compounds can thus be analyzed by virtual screening.

This method requires knowledge to either the crystal structure of the target or the chemical structure of known inhibitors or a natural ligand. Uses available compound databases, different compounds can be docked into matching protein-ligand complex. A summary of the steps involved in virtual screening is shown in Fig. 12.



Drug discovery and development: innovation/research-development/clinical development process.

(Fig. 12: steps involved in virtual (*in silico*) drug discovery and development: from gene to drug)

Validations are needed in both random and virtual screening, the enzyme target is cloned and lead compounds are collected, validations are done through enzymatic assay. In reality, most hit discovery campaigns involve both methods; direct screening and in-silico screening (17).

6.3 Lead optimization to select a clinical candidate

In order to get a ligand with high affinity to the protein, optimization of the ligand through repeatedly rounds if medical chemistry designs, synthesis and testing is needed. This is also referred to multi-parameter optimization. Once the micromolecular affinity has been established, the synthesis of the ligand can start and verification of the ligand can be tested on the actual protein, the so called pre-clinical stage (17).

6.4 Preclinical studies

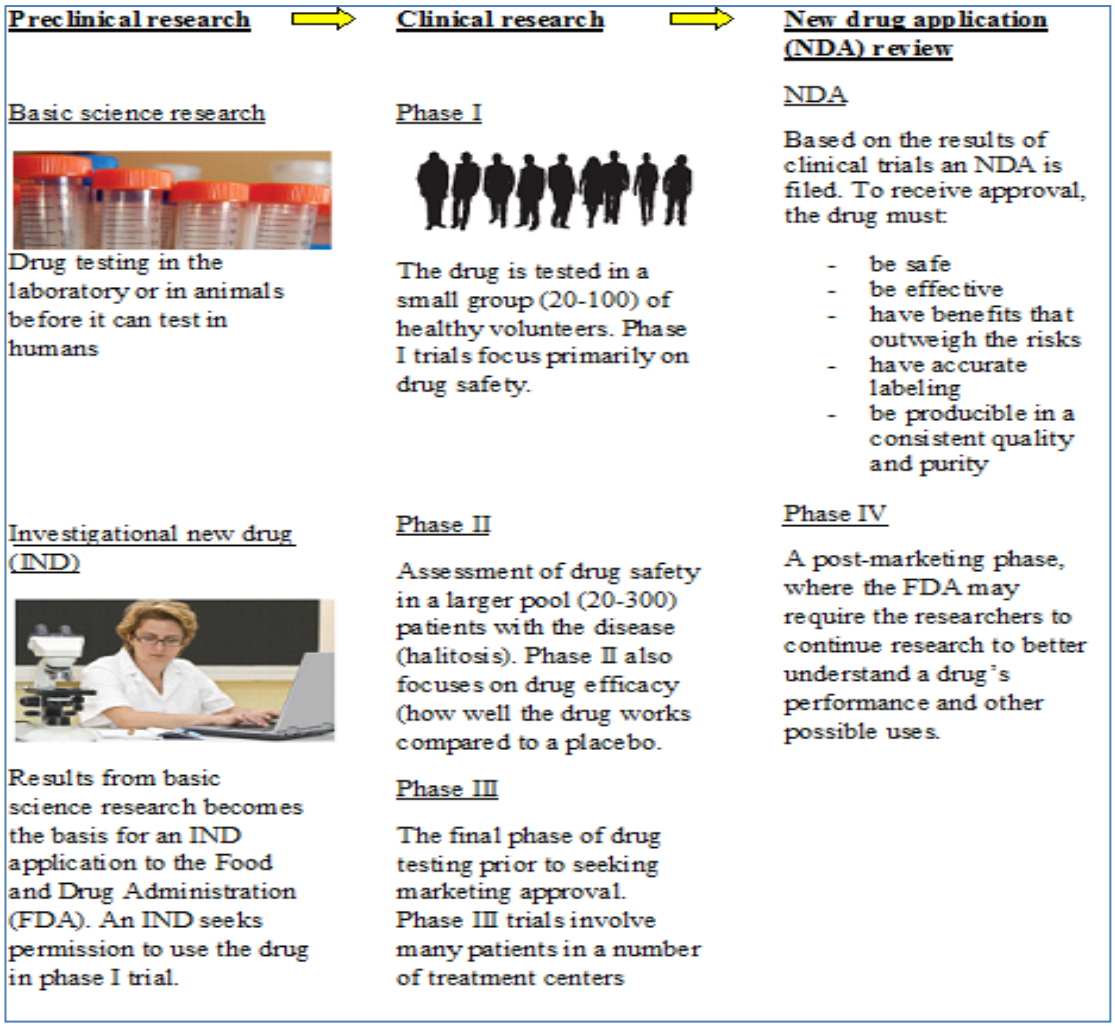
Preclinical models need to take account both of the molecular nature of the target and also of how the chemical compound will behave. Different models will be required for compounds targeting genetic dependency. Compounds that show promising activity in enzyme-assayed based assays will progress to in vivo animal studies. An example of these models used in preclinical studies is absorption, distribution, metabolism, excretion, toxicity properties evaluation (ADMET) (28).

Once a preclinical candidate has been identified, sufficient preclinical data have to be generated to support a clinical trial. For safety testing of small molecule drug candidates, generally the use of one rodent and one non-rodent species is recommended (17).

6.5 Clinical trials

Clinical trials for targeted drugs should be led by the biology and the clinical hypothesis. They should be designed to test a strong scientific hypothesis, i.e. particular drug acting on a specific molecular target is efficacious in patients with a particular type of genetic deviation or certain molecular feature. (17).

Phase I trials are often small studies designed to provide supporting information about a drug's pharmacokinetic parameters, dosing schedule, common side effects, tolerability, and toxicity, but are limited by design or other factors in their ability to demonstrate efficacy. Phase II and III trials are often larger studies designed to provide evidence on the overall risks and benefits of a drug (22). Figure 13 summarizes the steps involved in clinical trials.



(Fig. 13: Clinical trial phases, figure adapted from University of Connecticut Health Center, http://www.uchc.edu/patients/clinical_trials/pdfs/phases.pdf)

7. Easy Patient Sample Collection for Diagnostics

Recently a small company in Canada (DNA- GenotecK) has developed a simple kit both for the collection of biological samples such as saliva and DNA isolation at the same time.

In order to set a correct diagnosis of halitosis, sample of the patients' saliva would be required for isolating bacteria DNA from the specimen to check for oral bacteria causing halitosis presence. Nowadays methods in collecting saliva, the specimen need to be brought quickly to the nearest laboratory for analysis or prepared for storage is not optimal and is prone to mishandling of the samples, creating non-reliable data.

DNA-genotek's Oragene DNA (OG-500) facilitate the collection of samples from patient in an easy and efficient way, where the kit contains a tube with buffer already in the tube, once activated the buffer will be released into the tube. This ensures the sample is of optimal condition during shipping for analysis.

Below is a Table 9 taken from DNA-genotek's homepage, summarizing the advantages of their collection kit in comparison to traditional spit sampling.

Table 9: shows the advantages of Oragene DNA in comparison to other type of sampling. The ones highlighted are the ones of interest; saliva collection without the use of Oragene DNA vs. Oragene DNA collection kit (http://www.dnagenotek.com/ROW/products/OG500.html).

	Blood Collection	Oral Collection		
Attributes	Venous blood	Mouthwash	Buccal swabs	Oragene•DNA (OG-500)
Non-invasive collection	✗	✗	✓	✓
Standardized format for high-throughput processing	✓	✗	✗	✓
Specimen stability at room temperature	Days	Weeks	Days	Years
Low bacterial content	✓	✗† (up to 60% bacterial content)	✗† (up to 90% bacterial content)	✓† (median 11.8% bacterial content)
Median DNA yield	30 µg	35 µg	2 µg	110 µg
Sample size	1 mL	10 mL‡	1 swab	2 mL
Molecular weight	> 23 kb	> 23 kb	< 23 kb	> 23 kb
Shipping at ambient temperature	✗	✓	✓	✓
Full customization available	✗	✗	✗	✓

8. Discussion

The pathway of drug discovery from a gene to a drug is complex and consists of several stages (section 6 and Fig. 11):

- Target selection and validation
- Chemical hit and lead compound generation
- Lead optimization to select a clinical candidate
- Preclinical studies
- Clinical trials

There are two main methods in discovering potential inhibitor (lead compound) for VSC production by the responsible enzymes of the specific oral bacteria; 1- virtual (in-silico) screening and 2- random screening (High-throughput) (section 6.2). Table 10 summarizes the main differences between these two methods, using a previously validated enzyme targets.

Targets and target validations (section 6.1): in this thesis the targets are being identified because it is the main catalyst responsible for producing the volatile sulfur compounds. This is the most important steps for both methods for lead compound identification.

By applying homology modeling we can find amino acids sequence similarities of, enzymes from different bacterial families and species that share similar amino acid sequences, particularly in the active site domain. Comparing the nucleotide sequences would help identify the degree of relatedness of the studied enzymes as well as it would offer a framework, but to clone the gene coding for the enzyme as well as facilitating any needed subsequent genetic manipulations, such as site-directed mutagenesis, as needed for lead compound optimization. Biochemical and enzyme kinetics studies will help in setting up the screen and priorities any discovered lead-compound. Using enzyme x-ray crystallography will aid resolving the protein 3-D structure and this would help facilitate drug discovery by virtual screening.

Chemical hits, lead generation and optimization; following the virtual screening method, lead compound is found through online chemical compound library in e-format, where docking software are used in assessing the likelihood of the lead compound binding to the selected target. Plausible binding sites are identified; this is the site where a lead compound (inhibitor) may interact with the target (30).

Before entering clinical study, the lead compound is put through preclinical studies, where properties such as absorption, distribution, metabolism, excretion and toxicity are evaluated (ADME-model, see Fig. 12).

There are several stages in clinical trials; Fig. 13 divides the stages into phase I-IV. In Phase I, the drug (lead compound) is tested in a small group of volunteers that do not show any symptoms to halitosis. In phase II the drug is the assessed on its efficacy on a larger group of halitosis patients. In phase III trials will involve in even a larger pool of people with halitosis.

There are several limitations to this thesis. To find a target, a homology study using BLAST to identify similar enzymes in oral bacteria that produces volatile sulfur compounds are performed. From Tables (4-8), we see which oral bacteria have similar enzyme as the three

known enzyme in catalyzing sulfur containing amino acid to volatile sulfur compounds (VSC) leading to halitosis, meaning, in inhibiting these target will show a significant reduction in the production of VSC.

Table 2 shows which oral bacterium produces which volatile sulfur compound and from which substrate. Table 3 shows which the encoded enzyme in the production of VSC. I would have expected from using BLAST that many of the oral bacteria from Table 2 in the result list, but this are not the case. One reason is that not all listed oral species are sequenced and therefore not shown in the BLAST result. Secondly, many of the oral species from Table 2 are found in vivo, in patients with halitosis.

In the literature, *Fusobacterium nucleatum* is able to produce methyl mercaptan from methionine. Looking at chemical equations, MET-ase needs to be present to catalyze the reaction. BLAST-search was conducted where amino acid sequences of three known enzymes catalyzing the reaction in production of methyl mercaptan, hydrogen sulfide and then see which oral bacteria has similar sequences, from this we found *Fusobacterium nucleatum subsp.polymorphum* also contains enzyme L-cysteine desulfhydrase, turning L-cysteine to hydrogen sulfide. An interesting finding as it is not mentioned in the literature.

Another limitation is that when looking up each enzyme, it would have been expected that all oral bacteria mentioned in the literature to show in the BLAST result, but this is not the case. One reason is that not all oral bacteria are sequenced and many oral species which contain enzyme that are able to produce volatile sulfur compounds are linked to halitosis.

9. Conclusion

To my knowledge there are still no definite treatment to halitosis, though the market are swamp with products that promise a long lasting fresh breath, but these products do not eliminate halitosis. The active ingredients in these products have the potential to mask the bad smell by binding to sulfur and neutralize the gas (Zn-salts, chlorine dioxide) or even eliminate oral bacteria in a given time (Chlorhexidin, essential oils, triclosan, CFC) (Table 2). Many of these active ingredients give rise to unpleasant side effects, some products, might be effective against halitosis, but it is no near a cure. Chlorhexidin as an example has a bactericidal property, it will not only kill the oral bacteria causing halitosis, but it will also kill the normal flora found in the oral cavity. Our summarized approach is a target specific non-toxic by design and if succeed it will not be toxic to other oral bacteria, that do not possess enzymes releasing VSC.

To create a possible "cure", we need to identify a specific target that is of significant in the production of volatile sulfur compounds, and by inhibiting this target we will have an effective potential cure for halitosis.

The focus of this thesis has thus been looking at the specific chemical reactions and the enzymes that catalysis the production of sulfur compounds. Further, we have gone in depth and looked into the genetic sequences of three key enzymes; L-cysteine desulfhydrase, methionine gamma-lyase, L-methionine-alpha-deamino-gamma-mercaptomethane-lyase which is major contributors in the production of volatile sulfur compounds in oral bacteria (12, 14).

Using BLAST (Basic local alignment search tool), the nucleotide sequence of the three enzymes are compared against all sequenced bacterial species found in human. This enables us to survey if there are more oral bacteria that are significant in halitosis that contains similar enzymes. It is feasible to continue and follow-up by screening or virtual screening for the discovery of active compounds against the release of VSC. Similar to the principles of drug discovery, these lead compounds could be developed and optimized further, subjected to preclinical and clinical studies before launching to treat halitosis patients.

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