Future management and possible treatment of halitosis

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Acknowledgement

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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.
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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 desulphydrase, 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 desulphydrase, 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)

![Fig. 2: Right](http://www.jspsonline.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, trimethy-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).
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).

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

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-mercaptopmethane-lyase, responsible for methyl mercaptan (CH₃SH) 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 (H₂S), methyl mercaptan (CH₃SH), and to a lesser extent, dimethylmercaptan (CH₃SSCH₃). 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)

<table>
<thead>
<tr>
<th>Hydrogen sulfide from cysteine</th>
<th>Peptostreptococcus anaerobius</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Micros prevotii</td>
</tr>
<tr>
<td></td>
<td>Eubacterium limosum</td>
</tr>
<tr>
<td></td>
<td>Bacteroides spp.</td>
</tr>
<tr>
<td></td>
<td>Centipedia periodontii</td>
</tr>
<tr>
<td>Hydrogen sulfide from serum</td>
<td>Prevotella intermedia</td>
</tr>
<tr>
<td></td>
<td>Prevotella loescheii</td>
</tr>
<tr>
<td></td>
<td>Porphyromonas gingivalis (BANA positive)</td>
</tr>
<tr>
<td></td>
<td>Treponema denticola (BANA positive)</td>
</tr>
<tr>
<td></td>
<td>Selenomonas artemidis</td>
</tr>
<tr>
<td>Methyl mercaptan from methionine</td>
<td>Fusobacterium nucleatum</td>
</tr>
<tr>
<td></td>
<td>Fusobacterium periodonticum</td>
</tr>
<tr>
<td></td>
<td>Eubacterium spp.</td>
</tr>
<tr>
<td></td>
<td>Bacteroides spp.</td>
</tr>
<tr>
<td>Methyl mercaptan from serum</td>
<td>Treponema denticola (BANA positive)</td>
</tr>
<tr>
<td></td>
<td>Porphyromonas gingivalis (BANA positive)</td>
</tr>
<tr>
<td></td>
<td>Porphyromonas endodontalis</td>
</tr>
<tr>
<td>Others</td>
<td>Prevotella melaninogenica</td>
</tr>
<tr>
<td></td>
<td>Tanerella forsythia</td>
</tr>
<tr>
<td></td>
<td>Eikenella corrodens</td>
</tr>
<tr>
<td></td>
<td>Solobacterium moorei</td>
</tr>
<tr>
<td></td>
<td>Treponema forsythensis</td>
</tr>
<tr>
<td></td>
<td>Centipeda periodontii</td>
</tr>
<tr>
<td></td>
<td>Atopobium parvulum</td>
</tr>
</tbody>
</table>

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 (H₂S), methyl mercaptan (CH₃SH), and to a lesser extent, dimethylmercaptan (CH₃SSCH₃) (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)**

\[
\text{L-cysteine} \xrightarrow{\text{L-cysteine desulphydrase}} \text{H}_2\text{S} + \text{pyruvate} + \text{ammonia}
\]  (23)

**Equation II)**

\[
\text{Cysteine} \xrightarrow{\text{L-methion-γ-lyase}} \text{H}_2\text{S} + \text{pyruvate} + \text{ammonia}
\]  (11)

**Equation III)**

\[
\text{L-methionine} \xrightarrow{\text{METase}} \alpha\text{-ketobutyrate} + \text{methyl mercaptan} + \text{ammonia}
\]  (24)

From the equations the enzymes catalyzing the chemical reaction in the production of VSC are L-methionine-γ-lyase, L-cysteine desulphhydrase 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-\(\gamma\)-lyase, L-cysteine desulfhydrase, METase (L-methionine- alpha-deamino-gamma-mercaptopomethane-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-mercaptopmethylene-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.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Encoding gene</th>
<th>Volatile sulfur compound</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cysteine desulfhydrase</td>
<td><em>lcs</em></td>
<td>H(_2)S</td>
</tr>
<tr>
<td>L-methionine-gamma-lyase</td>
<td><em>megL</em></td>
<td>H(_2)S</td>
</tr>
<tr>
<td>L-methionine-alpha-deamino-gamma-mercaptopmethylene-lyase</td>
<td><em>mgl</em></td>
<td>Methyl mercaptan</td>
</tr>
</tbody>
</table>
5.1 Enzyme 1: L-cysteine desulphhydrase

Query: L-cysteine desulphhydrase
Source (organism): Aggregatibacter actinomycetemcomitans

Gene sequence

```
1  atgacatact atccagcaga ggcgttccga atcataaaagt tggacccgt tttcatattta
61  cccgagcag aacgcgaaat gcaatgaaa gaagcgggtt ataatacctt cttacttgat
121  tcaaacgtat cttcttaacc gtaacgcaaa ccaatgccgt gattttcttg gttgacccggc
181  caatggcag cagcgttacat ccttgggtcgt gattttcttg gttgacccggc
241  atgacatact atcgttccgac ccaccaagga
cgtgcggg ccaaatgaaa gaagcgggat ataatacctt cttacttgat
301  tcaaacgtat cttcttaacc gtaacgcaaa ccaatgccgt gattttcttg gttgacccggc
361  caatggcag cagcgttacat ccttgggtcgt gattttcttg gttgacccggc
421  atgacatact atcgttccgac ccaccaagga
cgtgcggg ccaaatgaaa gaagcgggat ataatacctt cttacttgat
481  tcaaacgtat cttcttaacc gtaacgcaaa ccaatgccgt gattttcttg gttgacccggc
541  caatggcag cagcgttacat ccttgggtcgt gattttcttg gttgacccggc
601  caatggcag cagcgttacat ccttgggtcgt gattttcttg gttgacccggc
661  caatggcag cagcgttacat ccttgggtcgt gattttcttg gttgacccggc
721  caatggcag cagcgttacat ccttgggtcgt gattttcttg gttgacccggc
781  caatggcag cagcgttacat ccttgggtcgt gattttcttg gttgacccggc
841  caatggcag cagcgttacat ccttgggtcgt gattttcttg gttgacccggc
901  caatggcag cagcgttacat ccttgggtcgt gattttcttg gttgacccggc
961  caatggcag cagcgttacat ccttgggtcgt gattttcttg gttgacccggc
1021  atgacatact atcgttccgac ccaccaagga
cgtgcggg ccaaatgaaa gaagcgggat ataatacctt cttacttgat
1081  atgacatact atcgttccgac ccaccaagga
cgtgcggg ccaaatgaaa gaagcgggat ataatacctt cttacttgat
1141  atgacatact atcgttccgac ccaccaagga
cgtgcggg ccaaatgaaa gaagcgggat ataatacctt cttacttgat
1201  atgacatact atcgttccgac ccaccaagga
cgtgcggg ccaaatgaaa gaagcgggat ataatacctt cttacttgat
1261  atgacatact atcgttccgac ccaccaagga
cgtgcggg ccaaatgaaa gaagcgggat ataatacctt cttacttgat
1321  atgacatact atcgttccgac ccaccaagga
cgtgcggg ccaaatgaaa gaagcgggat ataatacctt cttacttgat
```

//

Amino acid sequence

```
1  mttyypaepfr iksvepvsil pkaerekamk eagytflild skdvitmdl dsgtnamtsdr
61  qasimtggd eaygaagynf yhxvegqff kgklytqghq ggaenilri aiqpyggypq
121  mvyftrtyyq geanggqyf yhhdeahdat dldvppkggld klkklenline kgaenlacyc
181  lavgynl nagptvamkk vreltakhi kvfymgtcrv enayfekefe kygvdrqueik
241  lihmfsgqv gatcsgggttc agtvagtcctg tgaatctattc cctaatgaaa ccaatgccgt gattttcttg gttgacccggc
```
**BLAST result**

**Beyond the ten first hits**

<table>
<thead>
<tr>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query cover</th>
<th>E value</th>
<th>Ident</th>
</tr>
</thead>
<tbody>
<tr>
<td>L-cysteine desulfhydrase Fusobacterium nucleatum subsp. vanczecii 3_1_36A</td>
<td>801</td>
<td>801</td>
<td>98%</td>
<td>0.0</td>
<td>85%</td>
</tr>
<tr>
<td>L-cysteine desulfhydrase Fusobacterium nucleatum subsp. nucleatum subsp.</td>
<td>820</td>
<td>820</td>
<td>98%</td>
<td>0.0</td>
<td>85%</td>
</tr>
<tr>
<td>L-cysteine desulfhydrase Fusobacterium periodonticum 2_1_311</td>
<td>819</td>
<td>819</td>
<td>98%</td>
<td>0.0</td>
<td>85%</td>
</tr>
<tr>
<td>L-cysteine desulfhydrase Fusobacterium nucleatum subsp. polymorphus</td>
<td>816</td>
<td>816</td>
<td>98%</td>
<td>0.0</td>
<td>85%</td>
</tr>
<tr>
<td>L-cysteine desulfhydrase Fusobacterium nucleatum subsp. vanczecii 3_1_36A</td>
<td>816</td>
<td>816</td>
<td>98%</td>
<td>0.0</td>
<td>85%</td>
</tr>
<tr>
<td>L-cysteine desulfhydrase Citrobacter freundii sp. KTE32</td>
<td>809</td>
<td>809</td>
<td>98%</td>
<td>0.0</td>
<td>82%</td>
</tr>
<tr>
<td>L-cysteine desulfhydrase Citrobacter freundii sp. KTE15</td>
<td>807</td>
<td>807</td>
<td>98%</td>
<td>0.0</td>
<td>82%</td>
</tr>
<tr>
<td>L-cysteine desulfhydrase Citrobacter freundii sp. PIT31013_1TEL</td>
<td>806</td>
<td>806</td>
<td>98%</td>
<td>0.0</td>
<td>82%</td>
</tr>
<tr>
<td>Citrobacter freundii sp. KTE15</td>
<td>807</td>
<td>807</td>
<td>98%</td>
<td>0.0</td>
<td>82%</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Oral bacteria</th>
<th>Identity in amino acid sequence to L-cysteine desulfhydrase in <em>Aggregatibacter actinomycetemcomitans</em> (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusobacterium nucleatum subsp. animalis</em></td>
<td>86</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum</em></td>
<td>85</td>
</tr>
<tr>
<td><em>Fusobacterium periodonticum</em></td>
<td>85</td>
</tr>
<tr>
<td><em>Fusobacterium nucleatum supsp. vincentii</em></td>
<td>85</td>
</tr>
<tr>
<td><em>Treponema denticola</em></td>
<td>84</td>
</tr>
</tbody>
</table>

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

Gene sequence

```plaintext
1    aaaaatttaat ttattatttt tcaatattat tctttaaaaa atagaacctc tatatctatttt
36   ttaatgagtt cttttatattt tttcttctta gttatacaat taagttgaaa ataaagttttt
121  atagggaggt tttttatgta gcaaactctg taattgtatt aatttgggaac acccccttag
181  taataatattt taatattaat acttttggaa atgaaatata tgtaaaacta gaaggtcctaa
241  atctctgtag aagataaaaa acagagattg cctaaaaat gattgaaag gctgaaaaag
301  aaggttttaat tgattatttt ttttctttta gttatacaat taagttgaaa ataaagttttt
361  ggccttgcct gatagtgcac gtaaaacta ataatgata acatgcttac actgatctag
421  gctttttcctt ataatgata gataagttg aagaaagctt cctgatacct cttgatctag
481  gtttttttgg atggaagctg aattttaaga gataagttaaat attaagttt aatttgggaac
541  aatatatttt tcctaaaccc tctataaact taaataactc aaagctcact tatgaaacta
601  cagctagagg aatttttaag gatagtaaat aaagttgaa ataatattttg tattaatattg
ttttatttt gttatatatt gaatggtgat gaaactgtca aaaaaattca gattgc
661  gatagtgagtg agatagatttt aatggggccg aaagttgtaa gaaggtgcctt tattatat
721  aaatcttacc cggctgaacct gcgtctacct ctttacttta aaggggtat ataggtcctac
taatatttcg gttataataa atggtgagtt aatagttgaa ataatattttg tattaatattt
ttttatttt gttatatatt gaatggtgat gaaactgtca aaaaaattca gattgc
781  ataattttca ataatgata gataagttaaat attaagttt aatttgggaac
841  tagtctgtag aattttagtt gttgagagat gtaaagctca tattaatattt gattgc
901  gcttttttgg atggaagctg atgaaatttt ttttctttta gttatacaat taagttgaaa
961  aaatatttcg aatagttaaat attaagttt aatttgggaac
1021 gagaaaaata tctatcatag tctatagctt atataaaattattcactttcttctgc
```

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 desulphhydrase, [link to image])

Blast result:

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

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 desulphhydrase from fusobacterium nucleatum subsp.polymorphum and the oral bacteria fusobacterium periodonticum, which produces cysteine synthase.

**Delta Blast result**

![Fig. 4: showing the two comparing subjects; query = L-cysteine desulphhydrase and subject 1= cysteine synthase](image)

Even if the enzyme name is not the same; L-cysteine-desulphhydrase 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 desulphydrase is *Streptococcus anginosus*, the amino acid sequence are analyzed using BLAST (25).

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

**Amino acid sequence**

```
1  mrkynfqtap nrlshhtyw ketetdpqll pawiadmde vmpevkqaih dyaeqtvvgy  61
tyasellgq vidwexseh qyfdkedivf vegvvpaiis aigaftkegd avlinspyyp 121
dfarsvrlnn krlvynsle enlqfisqf qlekedivven vkllcsp npqwrivere 181
vlekighlqc khqvlvads ihqdltlfgh evhsfnitisp dfkfevals satktfng 241
tknysalen psrfaqfkr qllahnhev slgyisatet yrlygkpwdv lkdveleeniq 301
favdyfaka prlkvmqppq tylwlflfys ygltdelft llhdqavkl nrgadykgeg 361
elhrlniat pkplveeick rivhclpq
```

**BLAST result**

Beyond the ten first hits
**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.

<table>
<thead>
<tr>
<th>Oral bacteria</th>
<th>Identity in amino acid sequence to L-cysteine desulfhydrase in <em>s. anginosus</em> (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Streptococcus constellatus</em></td>
<td>96</td>
</tr>
<tr>
<td><em>Streptococcus intermedius</em></td>
<td>91</td>
</tr>
<tr>
<td><em>Streptococcus sp. oral taxon 056</em></td>
<td>77</td>
</tr>
<tr>
<td><em>Streptococcus gordonii</em></td>
<td>77</td>
</tr>
<tr>
<td><em>Streptococcus oralis</em></td>
<td>77</td>
</tr>
<tr>
<td><em>Streptococcus sanguinis</em></td>
<td>75</td>
</tr>
</tbody>
</table>
5.3 **Enzyme 2: L-methionine gamma-lyase**

Query: Methionine-gamma-lyase (methylene-γ-lyase)

Source (organism): *Fusobacterium nucleatum subsp. nucleatum* ATCC 25586

**Gene sequence**

CTTAACATTT CGTAAAGCTG GGTTGAAATC GTGACATCTG AATTCTTTTA TAATTTCTTT
CCTAATAATTC TACCTCTTCA CAGTGTACTA TGACAGTTTT TAGATTTAAAT AATTATTTTA
TAAATCTTGT TAAATATAA AAAAAATCA AATATAGTT AGGTAAATTA TGGAAATGAA AAAATCTGGT TTAGGAACAA
TTACTGCAGG GAGCTATGTGC AGGAGGAACT CTGCTATACA TGCAGGAACT TAAAAAATT
TAGTTGGTATG TTCCGAAACT CTGTTGAGAC CACCCAGCAT CTACTGAAAC TTTTAAAGGT TAAGATATGA
CTAGGTTGATC ATGCTGTGTC AATCTTCTTT TCTTTTCTTT TGCTTCTTAA ACAAGAAGCT

**Amino acid sequence**

```
1 memksgqgt taiahgtlkn lygtlampiy qtstfifdsa eggrrfale eayiytrlg
61 nptttvlenk iaaleegeag iamssmgai sstltvltka gdhvtdktl ygctfalmnh
121 gltrfgyvev fdtsnelveknamknkcaactaatattt tgaatggtag aagctgtgtg
181 vyvndtfatp ymgkpklgiv diivhsaaty lnhghdiag lvtvrqelad girfgvlkdm
241 tgvllqgeaa yyiirlkftf eiererhckn airtivdflnkpvekvevyp glethpgyei
301 akqkmnkdfga miselkkpgf eaqgtllnl klcsivslvg dtetlqhpa smthspytke
361 ereagitdg lvrlsvglen vediadleq 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*

<table>
<thead>
<tr>
<th>Description</th>
<th>Max score</th>
<th>Total score</th>
<th>Query cover</th>
<th>E value</th>
<th>Ident</th>
</tr>
</thead>
<tbody>
<tr>
<td>methionine gamma-lase <em>Fusobacterium nucleatum</em> → gb</td>
<td>812</td>
<td>812</td>
<td>100%</td>
<td>0.0</td>
<td>100%</td>
</tr>
<tr>
<td>methionine gamma-lase <em>Fusobacterium nucleatum</em> → gb</td>
<td>811</td>
<td>811</td>
<td>100%</td>
<td>0.0</td>
<td>95%</td>
</tr>
<tr>
<td>methionine gamma-lase <em>Fusobacterium sp., pratiaxen 8700</em> → gb</td>
<td>790</td>
<td>790</td>
<td>100%</td>
<td>0.0</td>
<td>96%</td>
</tr>
<tr>
<td>methionine gamma-lase <em>Fusobacterium nucleatum</em> subsp. <em>animalis</em> 4 81</td>
<td>789</td>
<td>789</td>
<td>100%</td>
<td>0.0</td>
<td>96%</td>
</tr>
<tr>
<td>methionine gamma-lase <em>Fusobacterium sp. CAG 2409</em></td>
<td>788</td>
<td>788</td>
<td>100%</td>
<td>0.0</td>
<td>96%</td>
</tr>
<tr>
<td>methionine gamma-lase <em>Fusobacterium nucleatum</em> → gb</td>
<td>788</td>
<td>788</td>
<td>100%</td>
<td>0.0</td>
<td>96%</td>
</tr>
<tr>
<td>methionine gamma-lase <em>Fusobacterium nucleatum</em> subsp. <em>virens</em> 3 1 36601 → ref</td>
<td>784</td>
<td>784</td>
<td>100%</td>
<td>0.0</td>
<td>96%</td>
</tr>
<tr>
<td>methionine gamma-lase <em>Fusobacterium nucleatum</em> → gb</td>
<td>774</td>
<td>774</td>
<td>100%</td>
<td>0.0</td>
<td>94%</td>
</tr>
<tr>
<td>methionine gamma-lase <em>Fusobacterium nucleatum</em> → gb</td>
<td>769</td>
<td>769</td>
<td>100%</td>
<td>0.0</td>
<td>93%</td>
</tr>
<tr>
<td>methionine gamma-lase <em>Fusobacterium periodonticum</em> → gb</td>
<td>768</td>
<td>768</td>
<td>100%</td>
<td>0.0</td>
<td>92%</td>
</tr>
<tr>
<td>methionine gamma-lase <em>Fusobacterium periodonticum</em> → gb</td>
<td>765</td>
<td>765</td>
<td>100%</td>
<td>0.0</td>
<td>92%</td>
</tr>
</tbody>
</table>
Table 6. Oral bacterial species with similar enzyme activity and sequence to methionine-γ-lyase in f. nucleatum subsp. nucleatum from first ten hits.

<table>
<thead>
<tr>
<th>Oral bacteria</th>
<th>Identity in amino acid sequence to methionine-γ-lyase in f. nucleatum subsp. nucleatum (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fusobacterium sp. oral taxon 370</td>
<td>96</td>
</tr>
<tr>
<td>Fusobacterium periodonticum</td>
<td>92</td>
</tr>
</tbody>
</table>

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: rofWP_005971502.1 | Length: 395 | Number of Matches: 1

<table>
<thead>
<tr>
<th>Score</th>
<th>Expect</th>
<th>Method</th>
<th>Identities</th>
<th>Positives</th>
<th>Gaps</th>
</tr>
</thead>
<tbody>
<tr>
<td>766 bits (1977)</td>
<td>0.0</td>
<td>Compositional matrix adjust.</td>
<td>364/395 (92%)</td>
<td>386/395 (97%)</td>
<td>0/395 (0%)</td>
</tr>
</tbody>
</table>

Query 1
MEMIKSGLIGTIAHSTILKONLYTLSLAMTPQSTFIIPDGESSAEQGGRBFALEAEASYIYRLG

Query 61
NPTTTTVLEK1AALNLEGEAASIAMSSGSGA1SSTNTVLKQAOGDRVVTKTLVYSTFFALNNH

Query 121
GLRFGVEVTVDSNLEEKVNAMEKHEKVVLYETPAKNLKVLELSK2AHYNETIL

Query 181
VIVONTFAPIHMOMX3LKPQGTV/VAISAYAYHNG5GVIAGLIGVLQGADQ1RFGVOLKEM

Query 241
TGAVLQEPQAYVIYTSK1FEMRHECD5KATIDFLMHRHKYKITEVYDSEP1VREYET

Query 351
ARKQMDQHCF1INSLK2CSLEAVSLGIDGELIHLFNASHSFYYKHE

Query 361
EREAAGITDGVLRSVGLENVDIADLEQGKEI

(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 mnrekeleklg faskqhags iknkygalat piyqtstfaf dsaeqgrcrf aleeeqiyt
61 rlngpttff eakaclegc eacmsaggi gyacsiwsi vnaqghiv ktygyctfafa
121 lhnglsrgy dvtyfdvtrdp envvkalkpn tkivyletpa npnmyldcia avskiahahn
181 peckvidnt ymtyrlqgrpl dlgadvhlhs atkylngbhd viaqfvgkgk efidqrvfng
241 vkdmtgstlg pfeaylirgrg mktdirmek hcaanaqvkve flekhpaves iafpgklksfp
301 qyelakqmk lcgamaafav kggleagktl insvxfatia vsgldaeli qhpsnhetls
361 ytepeaeed iaeglveisv gldedieila dldqalldlv k
```

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

<table>
<thead>
<tr>
<th>Oral bacteria</th>
<th>Identity in amino acid sequence to methionine-γ-lyase in <em>treponema denticola</em> (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Porphyromonas endotalis</em> (ATCC 35406)</td>
<td>75</td>
</tr>
<tr>
<td><em>Porphyromonas gingivalis</em></td>
<td>75</td>
</tr>
</tbody>
</table>
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 ttttataaatc gttttatgtt aatattataa tataaaaaca tcaaatatac taggaggtaa 
61 attatggaaa cgaaaaaata tggttttagga acaactgcta tacatgcagg aacatctaa 
121 aatttatagt gaactcttgc aatgccaata tatataactt ctacttttat atttgtccca 
181 gctgaacaag gttggaagag atttgctttt gaagaagctg gatatataa tacaagatctt 
241 ggaatccctca caacaacagtt tttgaataaat aatatttcgc aatgggagag aaggagaagct 
301 gctgttgctta cttcatcttgga atatctttaa tcatatgcag ctagtgacag tattttaaaa 
361 gcaggggacg atcgtggttttc tggataaact tttatggttt ctagcttttt tcattaagtgt 
421 catgtcctta caagagttcttt acttttgtgtt ctagcttttct ttatagagtctt 
481 gttaaaataag ctaatgaaatg aaataacaaga gttgttttact tggaaacacc tcgtctacc 
541 aatattttataa taagacagttt ctataacttt gctcaataac tccataaatct 
601 tttgttagttc tgcaaatataactt ccataatggc aaaaactttt aaatttaggtg 
661 gcagatatttgg ttgtttcacttt caacttttta aatatatttt ggtggtttct 
721 gtctttctttt taacactaataa ctagcttttc tataatatcg ctaggacttt cttataaatct 
781 atgacagggag catttttttac accacagatgtttattata ccatattgagg tattttaaaa 
841 ttttataaatc gatggttaaaagacatgcttt ctgcaaaattt gtagactggc 
901 aacacaccacc aataatcgg aagttgattt gctgtttaagt tttaccttttatttt 
961 atacacaaaaa aacattgaaag atgtttttactt gctgaaactgt acacactttt catttaaatct 
1021 tttgtaacatg tatttaacttt actataataac agtatataacttt gctccataacg ctgttttcat 
1081 ggaactcatg gcactcctttt aataacacttc gttcaacaccc gcaaatgttt aatgtttttact 
1141 gaagaacagat gatctgtttc aatagctgtc ggtttgattg gttagtcctgatt 
1201 aatgttgataa atataaatgc cagttttagga gatcagagttt gtttacagtttttat 
1261 tttattttacc ttcctttactt gttttggttt ttttctttctctttttttttt 
```

Amino acid sequence

```
1 metkkyglgt taihagtln lygtlapiy qtsttfidstsa eegqrgffkla eagyiytrlg 
61 npttttvlenk iaaleegaeaa vatsgmgatv sslltwltvka gdvvvtdkt1 yqctfalmch 
121 gltrfgiev ftmdtsnldev knakmmntrv vyletpapn nkivdleals kialhntpnt 
181 vivvntfatm ymqpkiklga divvnhavvyk inghqvdiag lvivktkelad qirfgilkm 
241 tgvllgppdqa yierrnmktf yiermerhkn akkkvveflnk hpkieryyyv hplethpghei 
301 akkmkdffga mifshakgfi egakttlnni klclsavlsl dtetlqjhpa smthspytke 
361 ereagidgq lvrlsvglgq veniadiqeq gkei 
```
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*
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

<table>
<thead>
<tr>
<th>Oral bacteria</th>
<th>Identity in amino acid sequence to MET-ase in <em>Fusobacterium nucleatum subsp. polymorphum</em>, ATCC 10953 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Fusobacterium periodonticum</em></td>
<td>96</td>
</tr>
<tr>
<td><em>Fusobacterium sp. oral taxon 370</em></td>
<td>92</td>
</tr>
</tbody>
</table>

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

(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$_2$S 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

(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 desulphhydrase, 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):

\[ 3\text{H}_2\text{S} + 2\text{BiCl}_3 \rightarrow \text{Bi}_2\text{S}_3 \downarrow + 6\text{HCl} \] (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).

<table>
<thead>
<tr>
<th>Details</th>
<th>Random screening (high-throughput screening)</th>
<th>Virtual screening (in-silico screening)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Requirements</td>
<td>Development of an assay to inhibit the activity of the enzyme(s) using non-toxic chemical libraries</td>
<td>Resolving the 3D-structure and modeling of the enzymes catalyzing the VSC if it is not known</td>
</tr>
<tr>
<td>Compound library</td>
<td>Pre-synthesize compounds, usually from corporate inventories</td>
<td>Compounds in e-format</td>
</tr>
<tr>
<td>Tools/hardware</td>
<td>Micro titer plates, plate controls, reagents, readout devices and analysis software</td>
<td>Structure- or ligand-based screening software; computing resources</td>
</tr>
<tr>
<td>Evaluation of hits</td>
<td>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</td>
<td>Scoring and ranking; visual inspection to detect presence of key interactions, chemical clustering</td>
</tr>
</tbody>
</table>
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.

(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.
7. Easy Patient Sample Collection for Diagnostics

Recently a small company in Canada (DNA-Genotek) 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](http://www.dnagenotek.com/ROW/products/OG500.html)).

<table>
<thead>
<tr>
<th>Attributes</th>
<th>Blood Collection</th>
<th>Oral Collection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Venous blood</td>
<td>Mouthwash</td>
</tr>
<tr>
<td>Non-invasive collection</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Standardized format for high-throughput processing</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Specimen stability at room temperature</td>
<td>Days</td>
<td>Weeks</td>
</tr>
<tr>
<td>Low bacterial content</td>
<td>✓</td>
<td>✓*</td>
</tr>
<tr>
<td></td>
<td>(up to 90% bacterial content)</td>
<td>(up to 90% bacterial content)</td>
</tr>
<tr>
<td>Median DNA yield</td>
<td>30 µg</td>
<td>35 µg</td>
</tr>
<tr>
<td>Sample size</td>
<td>1 mL</td>
<td>10 mL^2</td>
</tr>
<tr>
<td>Molecular weight</td>
<td>&gt; 23 kb</td>
<td>&gt; 23 kb</td>
</tr>
<tr>
<td>Shipping at ambient temperature</td>
<td>✓</td>
<td>✓</td>
</tr>
<tr>
<td>Full customization available</td>
<td>✓</td>
<td>✓</td>
</tr>
</tbody>
</table>
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 desulphhydrase, 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 desulphydrase, 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.
References

11) Kyosuke Suwabe, Yasuo Yoshida, Keiji Nagano and Fuminobu Yoshimura Identification of an L-methionine γ-lyase involved in the production of hydrogen sulfide from L-cysteine in Fusobacterium nucleatum subsp. Nucleatum ATCC 25586