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Thiobacilli of the Corroded Concrete Walls of the Hamburg Sewer System

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Thiobacilli were estimated in samples taken from the Hamburg sewer system at six sites showing different degrees of concrete corrosion. There was a marked enrichment of thiobacilli on the sewer pipe surface above the sewage level in comparison to the liquid phase. The highest number [10^8 thiobacilli (mg protein)$^{-1}$] was found at the site of the greatest corrosion. Ten isolates of the genus *Thiobacillus* were characterized and identified as *Thiobacillus neapolitanus*, *T. thiooxidans, T. intermedius* and *T. novellus*. Facultative chemolithotrophic bacteria predominated at sites of early corrosion, whereas *T. thiooxidans* was most abundant in severely corroded areas. The cell number of *T. thiooxidans* could be greatly decreased by aerating the sewage with pure oxygen.

INTRODUCTION

The rapid corrosion of sewage transport pipelines built from concrete was first reported by Olmstead & Hamlin (1900). Early investigators assumed that hydrogen sulphide, formed either by decomposition of organic material or by sulphate reduction in the sewage, escaped into the sewer atmosphere where it was oxidized to sulphuric acid. The oxidation was regarded as a purely chemical reaction catalysed by the concrete surface (Lea & Desch, 1936), and the corrosion resulted from sulphuric acid-induced changes in the concrete (Biczók, 1968; Thistlethwayte, 1972).

Parker (1945) isolated a highly acidophilic thiobacillus (*Thiobacillus concretivorus*, = *T. thiooxidans*) from corroded concrete sewers. He later isolated a second thiobacillus which also formed sulphuric acid (*Thiobacillus* X, = *T. neapolitanus*) and a third so-called 'thiobacillus' which oxidized thiosulphate without forming sulphuric acid (Parker, 1947; Parker & Jackson, 1965; Parker & Prisk, 1953). Therefore, concrete corrosion seemed to involve microbial as well as chemical processes (Schremmer, 1980).

The so-called 'hydrogen sulphide corrosion' has been observed predominantly in warm climates, but in recent years corrosion has become a serious problem in the Hamburg sewer system. This may be due to a high content of sulphur compounds in detergents, and an increase in the protein content of the sewage, which leads to a higher production of volatile sulphur compounds by amino acid degradation (Kadota & Ishida, 1972). New concrete sewers were corroded within a few years even if protected by a thin layer of epoxy resin. For that reason the Hamburg Municipal Drainage Department started an interdisciplinary investigation in 1978 on the course and prevention of the corrosion. The following studies were performed to determine which Thiobacillus species were present and what, if any, involvement they had in the corrosion process.

METHODS

Description of sampling sites and method of sampling. Samples were taken at various sites within the Hamburg sewer system. Site D1 is located at the outlet of a delivery pipe where strong currents and turbulence occur. The concrete surface, which was originally covered by a 200–300 μm coating of epoxy resin, is extensively corroded to a

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depth of several centimeters. Samples were taken from the loose material, which consisted mainly of gypsum. The concrete surface at sites D3, D4 and S14 is also covered by a thin epoxy resin coating, which is nearly intact at site D4, whereas it has many pustules and holes at sites D3 and S14. Samples were taken from the holes, which were mainly filled with gypsum, or by scraping off the surface of the epoxy coating. At sites D5, D6, and D7 the sewer surface is not coated with epoxy resin. The concrete is covered by a thin slime layer consisting of fungi, bacteria and dead organic material, and only minimal corrosion was observed. Samples were taken by scraping off the organic layer from the concrete surface. The pH of the concrete surface was determined by a surface electrode (Ingold) or indicator papers (Merck). All samples were collected with a sterile spatula and transferred to sterile plastic tubes. They were processed within 4 h after collection.

Media and growth conditions. The following media were used for the isolation and growth of the thiobacilli. (A) For *T. neapolitanus* the medium labelled *T. thioparus* of Vishniac & Santer (1957) was used at an initial pH of 6·6. (B) For *T. thiooxidans*, the S5 medium of Hutchinson *et al.* (1965), supplemented with 1% (w/v) $Na_2S_2O_3.5H_2O$, was used at an initial pH of 4·5. (C) For facultative chemolithotrophic thiobacilli the *T. intermedius* medium of Matin & Rittenberg (1971) supplemented with 0·5% (w/v) $Na_2S_2O_3.5H_2O$, 0·01% (w/v) $CaCl_2$ and 10^{-4} M-biotin was used at an initial pH of 6·8. (D) For *T. thioparus* the S7 medium of Hutchinson *et al.* (1965) was used. (E) For *T. denitrificans* the S8 medium of Hutchinson *et al.* (1967) was used under anaerobic conditions. (F) Medium A supplemented with 0·01% (w/v) yeast extract. (G) S0 medium of Hutchinson *et al.* (1965) supplemented with 0·02% (w/v) $Na_2S.9H_2O$. (H) S6 medium of Hutchinson *et al.* (1965). The solutions were autoclaved at 112 °C for 30 min. All cultures were grown at 30 °C and aerated by shaking or stirring.

Enumeration of bacteria. Samples were suspended in 10 ml sterile tap water and blended with a VirTis 45 homogenizer. The suspension was diluted in 10-fold steps to 10^{-8}. Sewage was diluted in the same way. For determination of total thiobacillus numbers, weakly acidophilic thiobacilli and strongly acidophilic thiobacilli were estimated by a five-flask (50 ml medium in 100 ml flasks) most probable number technique using media A and B, respectively. The inoculated flasks were shaken at 28 °C for 3 weeks. The presence of thiobacilli was determined in each flask by measuring thiosulphate consumption and acid production. The test was considered positive if more than 30% of the thiosulphate was consumed and the pH had decreased below 5 in medium A and below 2 in medium B. For differential determinations, *T. neapolitanus, T. thiooxidans* and facultative chemolithotrophic thiobacilli were estimated by a five-tube most probable number technique using media A, B and C, respectively. The tubes, each containing 3·5 ml, were shaken at 30 °C for 25 d. The test for *T. neapolitanus* was considered positive if the pH of medium A had dropped below 4; the test for *T. thiooxidans* was positive if the pH of medium B had dropped below 2. Since medium C supported the growth of *T. neapolitanus* as well as facultative chemolithotrophic thiobacilli, each tube showing turbidity and a drop in pH below 4·5 was streaked on medium C agar (1·5% agar) and incubated at 30 °C for 1 week. The test was considered positive if the colonies were transparent-yellowish and negative if they were white-opaque, which is typical for *T. neapolitanus* (Buchanan & Gibbons, 1974). For comparison, heterotrophic aerobes were estimated by viable counts on DEV-gelatin-agar (Merck). Since the samples at sites D1, D3 and S14 contained large amounts of inorganic corrosion products, sand and stones, the variation of cell numbers per wet or dry weight of sample material at these sites would be very high. Therefore, bacteria per mg protein are reported.

Isolation and identification of sulphur-oxidizing bacteria. Homogenized and diluted samples were streaked on agar plates of the media A to H. After incubation at room temperature for 2 weeks, single colonies were checked for thiosulphate oxidation in liquid medium. If the test was positive the isolate was checked for purity. Isolates were identified according to Hutchinson *et al.* (1969).

Determination of the G + C content of the DNA. DNA was prepared according to Marmur (1961), but with the following modifications. 1. For lysis, cells were first treated with lysozyme then with SDS for 1–2 h (concentrations as described by Marmur, 1961). 2. For deproteinization, no precipitation step with propan-2-ol was used. 3. After treatment with RNAase, the suspension was incubated with 0·22% (w/v) Pronase in 0·15 M-NaCl (pH 5·0) for 60 min at 37 °C. The G + C content of DNA preparations was determined according to Marmur & Doty (1962). A Gilford photometer (type 240) with thermoprogrammer (type 527) was used for determination of T_m values. DNA solutions were heated in a specially designed cuvette with four Teflon-coated melting positions. The DNA of *Escherichia coli* K12 was used for calibration.

Electron microscopy. Cells were embedded and ultrathin sections were examined in the electron microscope as previously described (Bock & Heinrich, 1969).

Analyses. Protein was determined by the Lowry method. Thiosulphate was determined by titration with 0·0025 M-iodine solution after the addition of a zinc iodide/starch solution and phosphoric acid (Deutsche Einheitsverfahren zur Wasseruntersuchung, 1966).

RESULTS

Counts of thiobacilli and heterotrophic bacterial populations at the different sampling sites are presented in Table 1. Although samples at any one sampling site showed a great deal of

Table 1. *The number of thiobacilli and heterotrophic bacteria in samples from sewer walls, sewage, and garden soil*

The data are mean values from 4–8 samples. The variation of cell numbers between samples at a given sampling site was ±1 tenth power.

Sampling site		Degree of corrosion*	No. of cells (mg protein)$^{-1}$		Protein [mg (g wet wt)$^{-1}$]
			Thiobacilli	Heterotrophic bacteria	
D1	wall	extensive	1×10^{8}	4×10^{7}	2·8
	sewage	—	4×10^{4}	8×10^{7}	0·20
D3	wall	moderate	2×10^{7}	2×10^{7}	2·0
	sewage	—	2×10^{4}	2×10^{7}	0·18
S14	wall	moderate	2×10^{6}	1×10^{8}	2·2
	sewage	—	3×10^{3}	2×10^{8}	0·25
D7	wall	slight	3×10^{6}	1×10^{8}	ND
	sewage	—	2×10^{3}	3×10^{6}	0·45
D5	wall	minimal	4×10^{7}	9×10^{6}	11·0
	sewage	—	2×10^{4}	3×10^{8}	0·20
D6	wall	minimal	7×10^{5}	6×10^{7}	15·3
	sewage	—	5×10^{3}	1×10^{8}	0·25
Garden soil		—	9×10^{3}	2×10^{6}	7·8

ND, Not determined.

* Degrees of corrosion according to M. Lohse, C. F. Seyfried, H. Nehlskamp & H.-J. Wierig (personal communication).

variation the data clearly indicated a strong enrichment of thiobacilli on the concrete walls in comparison to sewage or garden soil. Furthermore, on highly corroded concrete walls the thiobacilli had reached the same population level as the heterotrophs.

Identification of thiobacilli

Ten isolates were subjected to identification procedures (Table 2). All were Gram-negative, rod-shaped, 2–4 μm long, and 0·5–0·8 μm wide. All isolates except strain 42 were motile by means of a single polar flagellum. All isolates except strain D16 had carboxysomes. Reduced sulphur compounds were oxidized by all isolates but the amount of acid produced varied. No isolate was able to oxidize thiocyanate, dithionate and iron, or to grow anaerobically (test conditions according to Hutchinson *et al.*, 1969). Isolates 33 and 55 were identified as *T. neapolitanus* because of their G + C content of 54–55% (Jackson *et al.*, 1968), the final pH of 3·0 reached in liquid media, their inability to oxidize thiocyanate, and their insensitivity to high ionic strength (Hutchinson *et al.*, 1969). Isolates K6, K11 and K16 are strains of *T. thiooxidans* because of the final pH of 1·0, their inability to oxidize Fe^{2+} (Hutchinson *et al.*, 1969), and their relatively low G + C content (K6, K16). Isolates D5, D14, and K12 were identified as *T. intermedius* because of their ability to grow heterotrophically (London, 1963), their sensitivity to high ionic strength (Hutchinson *et al.*, 1969) and the presence of carboxysomes (Shively *et al.*, 1970). Isolate D16 was identified as *T. novellus* because it was unable to grow on sulphur as the sole energy source (Hutchinson *et al.*, 1969) and did not possess carboxysomes (Shively *et al.*, 1970). The taxonomic position of strain 42 is uncertain. It resembles *T. neapolitanus*, but has a lower G + C content of 52·9% and is less resistant to high ionic strength (Hutchinson *et al.*, 1969). All attempts to isolate additional species of thiobacilli by means of selective media failed, i.e. *T. thioparus*, *T. denitrificans* and *T. ferrooxidans* were not found. However, several pure cultures which oxidized thiosulphate, but not elemental sulphur, in the presence of 0·01% (w/v) yeast extract (medium F) were obtained. This oxidation, measured as thiosulphate consumption, led to a pH increase in the growth medium, which is typical for the formation of tetrathionate from thiosulphate (Trudinger, 1967). As cell numbers were identical in media with or without thiosulphate, the bacteria probably do not obtain energy from the oxidation process. Similar bacteria have been described as '*Thiobacillus trautweinii*' (Trautwein, 1921; Trudinger,

Table 2. *Identification of thiobacilli*

		Characteristics of isolates									
Medium*	Criterion	33	42	55	K6	K11	K16	D5	D14	K12	D16
S6/S5	Final pH	3·0	3·0	3·0	1·1	1·2	1·2	2·5	2·4	2·5	2·7
S6/S5	Thiosulphate oxidation (%)	100	100	100	90	90	90	100	100	100	100
S0 + 6% (w/v) thiosulphate	Thiosulphate oxidation (%)	96	40	96	10	10	10	0	0	0	0
S6/S5 + 4 (w/v) phosphate	Inhibition	–	+	–	–	–	–	+	+	+	+
S6/S5 + 5% (w/v) NaCl	Inhibition	–	+	–	+	+	+	+	+	+	+
Sulphur	Final pH	3·0	3·0	3·0	1·0	1·5	1·1	2·9	2·9	2·9	NG
Nutrient agar plate	Growth	–	–	–	–	–	–	+	+	+	+
Thiosulphate agar	Sulphur deposition	+	+	+	+	+	+	–	–	–	–
S0 + 0·5% (w/v) tetrathionate	pH change	+	+	+	+	+	+	+	–	+	+
S0 + 0·02% (w/v) sulphide	Growth and sulphide oxidation	–	–	–	–	–	–	–	–	–	–
S0 agar + 50 p.p.m. dimethyl disulphide in the gas phase†	Growth	–	–	–	–	–	–	ND	ND	ND	ND
	T_m (°C)‡	92·0	91·1	91·6	91·2	ND	91·5	96·6	96·7	96·5	96·7
	% G+C	55·1	52·9	54·2	53·2	ND	53·9	66·4	66·7	66·1	66·7

NG, No growth; ND, not determined.

* Media were used according to Hutchinson *et al.* (1969): S6/S5 = mineral solution + 1% (w/v) thiosulphate, initial pH 6·6 (S6 medium) and 4·5 (S5 medium); S0 = mineral salt solution without substrate; sulphur = S6/S5 + 1% (w/v) sulphur. For strains D5, D14, K12, and D16, 10^{-4} M-biotin was added to all media.

† Plates were inoculated with the isolated strains and incubated in an atmosphere containing 50 p.p.m. dimethyl disulphide.

‡ Melting point of the DNA according to Marmur & Doty (1962).

1967), but are no longer regarded as thiobacilli. One of the isolated strains was characterized and identified as a *Pseudomonas* sp.

Occurrence of thiobacilli at different degrees of concrete corrosion

The thiobacilli varied with different degrees of corrosion and appeared to be related to the acidity present (Fig. 1). *Thiobacillus thiooxidans* predominated in areas of extensive corrosion with low pH values of 1 to 2. At moderate corrosion levels with a pH between 5 and 6, *T. neapolitanus* and the facultative chemolithotrophic thiobacilli were present in similar numbers, whereas only a small part of the population consisted of *T. thiooxidans*. The facultative chemolithotrophic thiobacilli predominated in areas of low corrosion (pH 6) when excess organic material was present.

Effects of oxygen supply to the sewage

From June 1980 to January 1981, the sewage at the sampling site D1 was supplied with pure oxygen. During this period, the pH of the concrete surface increased from 2 to 5 to 6, whereas the number of *T. thiooxidans* decreased from 10^7 to about 10^1 cells (mg protein)$^{-1}$ (Fig. 2). The cell numbers of *T. neapolitanus* and facultative chemolithotrophic thiobacilli did not decrease significantly. After the oxygen supply was turned off, the pH of the concrete surface dropped below 4 and the cell number of *T. thiooxidans* increased to 10^6 (mg protein)$^{-1}$ within 5 months.

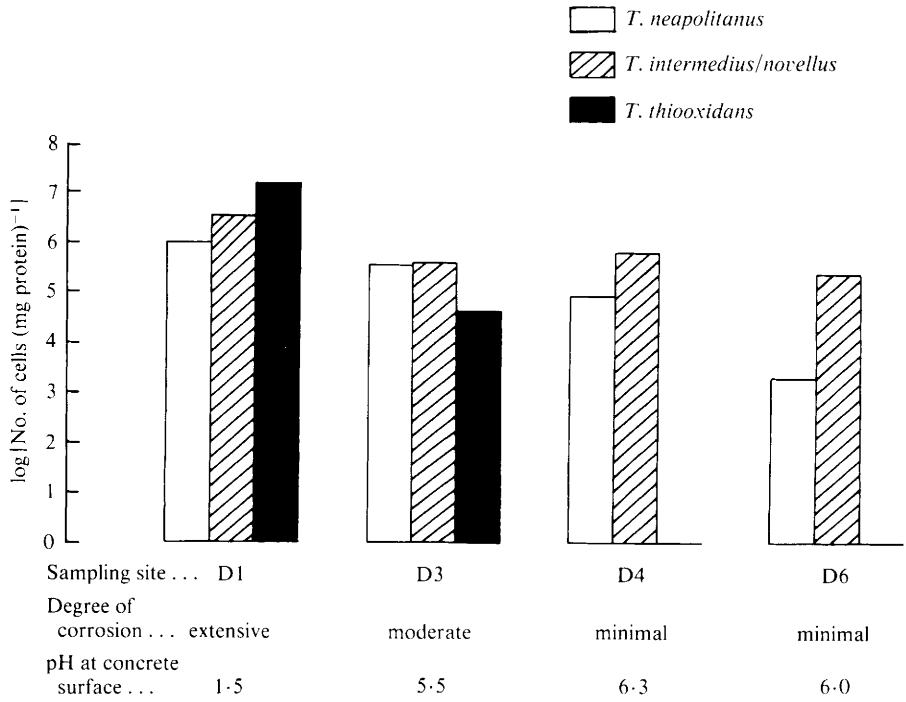

Fig. 1. Distribution of three thiobacillus types at sites exhibiting different degrees of concrete corrosion. The cell numbers and pH values are mean values from 3–6 samples.

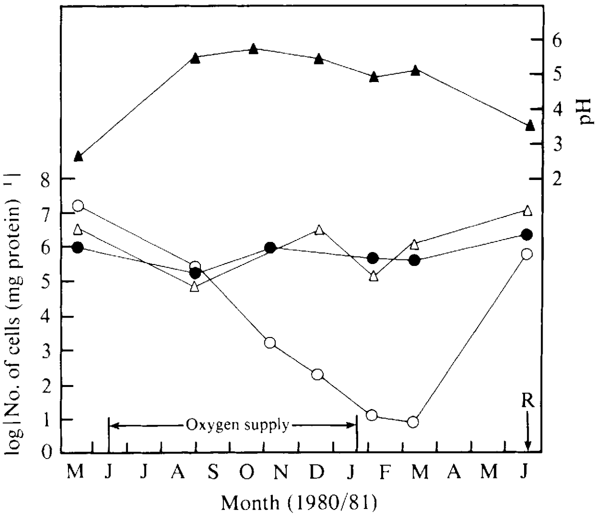

Fig. 2. Development of the thiobacillus flora at sampling site D1. ○, *T. thiooxidans*; ●, *T. neapolitanus*; △, facultative chemolithotrophic thiobacilli; ▲, pH of the concrete surface; R, replacement of the corroded sewer. The data are mean values from 3 samples.

DISCUSSION

The data indicate that thiobacilli can be found on the corroded walls of Hamburg sewers. The results of the ecological studies could be interpreted as a successive development of the different thiobacilli during the corrosion process. At the beginning of corrosion, facultative chemolithotrophic thiobacilli like *T. intermedius* and *T. novellus* which were able to grow in neutral or slightly alkaline environment predominated. As the corrosion proceeded and the pH value at the concrete surface dropped to less than 6 the number of *T. neapolitanus* increased. If the pH dropped below 5, *T. thiooxidans* started growing, causing further acidification. These results are partly in agreement with those of Parker (1947), Parker & Prisk (1953) and Parker & Jackson (1965) obtained for the Melbourne sewerage system. These authors isolated large numbers of *T. thiooxidans*, *T. neapolitanus* and organisms which convert thiosulphate to tetrathionate resulting in an increase in pH value ('M-strains', '*T. trautweinii*'). In contrast to our results, however, they did not isolate facultative chemolithotrophic thiobacilli, the predominant group at sites of minimal corrosion in Hamburg sewers. Furthermore, we could not isolate *T. denitrificans* and *T. thioparus*, which were found in relatively high numbers in Melbourne sewers. The differences in the compostion of the thiobacilli appear to be due to the environment. In corroded concrete high amounts of sulphate and sulphuric acid were present, but no organic acids could be found by gas chromatography and mass spectrometry (unpublished results). The high numbers of thiobacilli in the corrosion products indicate that the production of sulphuric acid is mainly a microbial process. In contrast to the Melbourne sewers, the atmosphere of the Hamburg sewer system contained various organic polysulphides and elemental sulphur and only negligible concentrations of hydrogen sulphide could be detected (König *et al.*, 1980). Our isolates were unable to utilize sulphide and dimethyl disulphide for cell growth. These results are in contrast to those of Sivelä & Sundman (1975) who reported growth of thiobacilli on organic polysulphides. These authors used unidentified isolates which may have been adapted to high concentrations of organic polysulphides present in the environment of a cellulose mill. It should be noted that hydrogen sulphide as well as organic polysulphides are oxidized to elemental sulphur in the presence of oxygen by a purely chemical process. Incubating concrete blocks in an atmosphere containing 10–50 p.p.m. hydrogen sulphide in the absence of thiobacilli resulted in the deposition of high amounts of elemental sulphur on the concrete surface; after inoculation of the blocks with a mixture of the strains 33, 55, K6, K16, D16 and K12, the pH on the concrete surface dropped from 9 to 1 within 6 months (unpublished results). Since elemental sulphur could be detected on the sewer walls, and is a very good substrate for thiobacilli, it seems likely that elemental sulphur and not hydrogen sulphide or organic polysulphides is the main energy source of thiobacilli on sewer walls of the Hamburg sewer system. The inhibition of growth of the strongly acid-producing *T. thiooxidans* by enrichment of the sewage with oxygen (Fig. 2) can be interpreted as follows. By inhibition of anaerobic processes (sulphate reduction) the formation of volatile sulphides, which – directly or indirectly – are the main energy source of thiobacilli, was greatly diminished. When the production of sulphuric acid decreased, the pH of the concrete surface gradually increased because of the buffering action of the cement. Consequently, there was a decrease in the cell numbers of the strongly acidophilic *T. thiooxidans*, which has a pH optimum of 2–3. After the oxygen supply was turned off, anaerobic conditions could gradually be re-established in some parts of the sewer. Acid production could start again, the pH of the concrete surface dropped, and consequently *T. thiooxidans* began to multiply.

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