Presence of *Acinetobacter* species among the predominant bacteria found in a contaminated metal-working fluid

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The distribution of *Acinetobacter* species among the bacteria isolated from an unusually heavily contaminated petroleum-based water-soluble metal-working fluid used in a large North American automotive machining operation was investigated. It had been found previously[1] that, in laboratory cultures with metal-working fluids as the sole carbon source, these species grew more rapidly and to much higher densities than any other types of bacteria isolated from this system. Thus, it was expected that they would dominate the population and would be difficult to control. It is shown here that, contrary to expectation, over a one-year period *Acinetobacter* species usually accounted for only a few percent of the total population. Factory fluids did not contain substances that selectively inhibited the growth of these species. In mixed-cultures grown in the laboratory with metal-working fluid as the carbon source, *Acinetobacter* species accounted for less than 1% in the initial inoculum, nearly 70% during the mid-exponential-phase, and only a few percent in the stationary-phase. These experiments suggest, as a working hypothesis, that the *Acinetobacter* species 'govern' the contamination in the sense that they first colonize the fluid and their presence is necessary to maintain the other strains.

Keywords: *metal-working fluids, bacterial contamination,* Acinetobacter *species*

Bacterial densities in contaminated metal-working fluids may range from less than 10^6 to greater than 10^9 colony-forming units (CFU)/ml[1-4]. The bacteria are of concern because they may, in many cases, cause metal-working fluid degradation and, under some circumstances, they may be opportunistic pathogens. Thus, a major goal in the maintenance of metal-working systems is minimizing bacterial densities.

The present authors have been investigating the causes of very high bacterial densities ($> 10^9$ CFU/ml) in the metal-working fluid of a large machining operation in a North American factory of the Ford Motor Company. The fluid ingredients that were present in concentrations high enough to provide the amounts of carbon necessary to support the bacterial growth were naphthenic (cycloparaffinic) petroleum oils, oleic and linoleic acids and petroleum sulphonates. Although many investigators had shown that bacteria commonly found in metal-working fluids might be expected to grow to densities as high as 10^7 CFU/ml[5], no evidence could be found in the literature suggesting that these bacteria were capable of growth to 10^9 CFU/ml with these materials as the carbon source[6,7]. Thus, as described previously[1], the components of the fluid

were studied as carbon sources for bacteria isolated from the fluid and the predominant bacteria were identified. Among the bacteria isolated from the fluids, species were found of the genus *Acinetobacter* which grew rapidly (24 hours) and to high density (10^9 CFU/ml) with fluid components or intact fluids as the sole carbon sources[1]. The other bacteria, mostly *Pseudomonas* strains, grew much slower and to densities 10 to 1000 times lower than the *Acinetobacter* strains under the same growth conditions. To the knowledge of the present authors, *Acinetobacter* species had not been previously recognized in contaminated metal-working fluids, even though they are common soil bacteria and one of their main characteristics is prolific growth on certain long chain alkanes ($C_{10}-C_{20}$) as sole carbon sources[6,8,9].

The observations just summarized led to the expectation that most of the bacterial strains isolated from the fluid would be species of *Acinetobacter*. This expectation, however, was based on experiments with pure cultures. In view of the well-recognized difficulties in reaching conclusions about mixed cultures from experiments on pure cultures, these expectations were tested by determining the proportion of *Acinetobacter* species in samples obtained over a one-year period. The species distribution in mixed cultures propagated in the laboratory with metal-working fluid as the carbon source was also investigated. The results of these experiments are reported here.

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Materials and methods

Sampling of contaminated fluids and isolation of pure cultures from them were carried out as described previously[1]. Growth experiments in liquid cultures with fresh metal-working fluid, obtained from the factory bulk reservoir, as the sole carbon source were carried out in 5% (v/v) fluid added to sterile mineral-salts solution[10] and incubated at 27°C in a shaker-incubator.

Used fluid from the factory was sterilized by centrifuging (at 9000 g) twice to remove as many of the bacteria as possible and then pasteurizing it for 15 minutes (at 78°C). Sterility was confirmed by incubating the samples for three days at 27°C and plating 100 μl aliquots.

A defining trait of the *Acinetobacter* genus is a negative oxidase test[9,11]. The oxidase reagent, a 1% solution of N,N,N'N'-tetramethyl-*p*-phenylenediamine, was prepared fresh daily. A portion of a colony was spread over a patch of filter paper using a wooden stick and a drop of oxidase reagent applied over the patch. If a dark purple colour developed within 10 seconds the bacterium was oxidase positive.

The proportion of *Acinetobacter* species was estimated by diluting contaminated fluid and plating it on nutrient agar so as to obtain plates with between about 50 and 100 colonies. Every colony on the plate was then characterized. Three samples from the factory were analysed by first testing every pure colony for oxidase activity and then testing every oxidase-negative isolate with a commercial taxonomic test kit — the 'Rapid NFT Test' (API Analytab Products, Plainview, New York, USA). For four samples, only the oxidase test was carried out, which yielded an upper limit to the proportion of *Acinetobacter* species. This estimate was based on our experience that at least 90% of the oxidase-negative isolates from this system were *Acinetobacter* species. For three other samples, the estimate of the upper limit to the proportion of *Acinetobacter* species was based on the number of colonies that grew with hexadecane vapour as the carbon source. This method was based on the observation that every *Acinetobacter* species isolated (about 65 isolates) formed robust colonies when streaked on mineral-salts-agar plates and incubated with hexadecane vapour (50 μl deposited on a piece of filter paper placed on the cover of an inverted petri plate) as the only carbon source. Conversely, out of 100 randomly selected colonies that grew on hexadecane vapour, only two were oxidase positive (i.e. they were not *Acinetobacter* species). Plates cultured without hexadecane served as a control to detect the occasional strain capable of growth on agar as the carbon source.

Results

The proportion of *Acinetobacter* species was estimated in ten samples of metal-working fluid obtained at irregular intervals for about one year. The first sample was obtained during a period when the densities of bacteria were consistently greater than 10^9 CFU/ml. The other nine samples were obtained after a new maintenance programme was started. This maintenance programme involved repairing and improving pumps and filters, minimizing leaks of hydraulic fluids, cleaning and monitoring and maintaining the fluid pH and emulsion stability. The maintenance programme did not involve any changes in the metal-working fluid. Biocides were not used at any time.

Table 1. Proportion of *Acinetobacter* species among metal-working fluid bacteria

Sample date	Total density (CFU/ml)	Number colonies isolated	% *Acinetobacter* species
10-23-85	2×10^9	117	18%*
12-12-85	9×10^7	85	7%*
1-15-86	6×10^7	57	25%*
5-13-86	5×10^7	81	< 1%†
5-20-86	2×10^7	54	< 2%†
7-1-86	6×10^7	60	< 2%†
9-20-86	2×10^8	81	< 1%†
9-12-86	4×10^7	–	< 0.2%‡
9-19-86	2×10^7	–	< 0.5%‡
10-10-86	4×10^7	–	< 4.0%‡

*Commercial taxonomic kit used for identifications of oxidase-negative colonies.

†Only oxidase test was done. Percentages are upper limits because not all oxidase-negative cultures were *Acinetobacter* species.

‡These samples were analysed by diluting and plating for growth on hexadecane vapour. These are upper limits because a small percentage of the bacteria grew on hexadecane, but were not *Acinetobacter* species (see section of text on materials and methods).

The proportion of *Acinetobacter* species ranged from less than 1% to about 25% (see Table 1). Moreover, the total bacterial densities decreased 10 to 100-fold following the start-up of the aforementioned maintenance programme. Thus *Acinetobacter* species did not make up the entire population and did not persist at near 10^9 CFU/ml, despite their copious growth in laboratory cultures with metal-working fluid or its components as the carbon source.

One hypothesis to explain why *Acinetobacter* species did not dominate the bacterial populations was that the factory reservoirs contained a substance that selectively inhibited the growth of *Acinetobacter*. This hypothesis was tested with two experiments. In the first experiment, the growth rates were determined of three different *Acinetobacter* species, isolated previously from the system, with sterilized (see the previous section) used-fluid (50% v/v) as the only carbon source. For all three strains, the density after 24 hours, starting from an initial inoculum of about 10^3 CFU/ml, was about 2 × 10^9 CFU/ml — the same as observed for 5% v/v fresh fluid as the carbon source.

The second experiment tested for the possible presence of inhibitors that were unstable under the pasteurization conditions used in the first experiment. An unsterilized sample from the plant was mixed with an equal volume of mineral-salts containing 2% agar and poured into a petri plate. *Acinetobacter* species were then added, to a density of 5 × 10^2 CFU/ml, to 2 ml of 'top-agar', prepared with 0.6% low-melting point (27°C) agar instead of 2% regular agar. This 2 ml of top-agar was then poured onto the surface of the solid agar containing the contaminated fluid. In this way, the *Acinetobacter* colonies were distinctly visible in the top-agar layer despite the opacity of the bottom layer of agar. The number of *Acinetobacter* colonies observed in the presence of the sterilized metal-working fluid was the same as that in the presence of the unsterilized fluid. Thus, it was concluded that the used fluids did not contain any substances that inhibited the growth of *Acinetobacter* species.

Table 2 Proportion of *Acinetobacter* species in mixed cultures grown in the laboratory

| | Number of colonies | | | | | | | |
| | Isolated* | | Oxidase negative | | Growth on hexadecane† | | % *Acinetobacter* | |
Time after inoculation	Experiment No. 1	No. 2	Experiment No. 1	No. 2	Experiment No. 1	No. 2	Experiment No. 1	No. 2
0 hours‡	55	39	2	6	0	1	< 2%	2%
24 hours	47	25	41	15	38	15	77%	60%
48 hours	56	50	30	25	30	17	53%	50%
72 hours	112	44	31	29	28	27	25%	61%
144 hours	–	32	–	10	–	0	–	< 3%

*Number of colonies that grew on the plates; every colony was tested for oxidase activity and for growth on hexadecane vapour.

†Colonies that were both oxidase negative and grew on hexadecane vapours (48-hour incubation time).

‡0-hour sample obtained by removing aliquot from medium immediately after it was mixed.

Mixed cultures grown in the laboratory on medium with 5% metal-working fluid as the carbon source were then investigated. The mixed culture was started by adding 200 μl of contaminated fluid from the factory to 20 ml of medium. At various times, the proportion of *Acinetobacter* species was estimated based on the number of colonies that were both oxidase negative and grew on hexadecane vapour (see the previous section). The results are presented in Table 2. The percentage of *Acinetobacter* species in the fluid as it came from the factory was less than 2%, but after 24 hours, when the cultures were in the exponential growth phase, the *Acinetobacter* species accounted for most of the population. The proportion of *Acinetobacter* species then declined (after 72 hours in experiment 1 and after 114 hours in experiment 2) when the cultures were, presumably, in the stationary phase. These results suggest that the low percentage of *Acinetobacter* species in the samples from the factory may be characteristic of mixed cultures in the stationary phase.

As noted above (see Table 1), the bacterial densities in the fluids after the start of the maintenance programme were usually about 5×10^7 CFU/ml compared with near 10^9 CFU/ml before. The simplest explanation for the decline in total densities is a reduction in the concentration of one or more nutrients. The most likely limiting nutrient was nitrogen. Nitrogen starvation was tested for by growing mixed cultures and pure *Acinetobacter* strains on media with and without added inorganic nitrogen (see Table 3). The findings suggest that the available nitrogen may limit the total population of bacteria in the factory samples to between 10^7 and 10^8 CFU/ml.

The pH of the fluid in the factory ranged between about 7.9 and 8.5, but laboratory growth experiments carried out

Table 3 Tests for nitrogen sources in metal-working fluids

| | Bacterial density (CFU/ml) | | | |
	0 h	24 h	48 h	72 h
Acinetobacter species in:				
Salts* + N + acetate	6×10^2	3×10^8	1×10^9	–
Salts + tap water + acetate	6×10^2	3×10^7	8×10^7	–
Salts + MWF + acetate	5×10^2	2×10^5	1×10^7	–
MWF + tap water	5×10^3	1×10^6	7×10^7	–
MWF + tap water + N	4×10^3	4×10^9	3×10^8	–
Mixed culture in:				
MWF + tap water	3×10^2	6×10^4	7×10^7	2×10^8
MWF + tap water + N	3×10^2	1×10^4	6×10^8	6×10^7

*'Salts' is the mineral-salts solution described in text, but with nitrogen omitted. Where indicated, potassium acetate (0.1%) was added as a carbon source. Metal-working fluid (MWF) concentration was 1%. 'N' indicates that ammonium sulphate (0.1%) was added as a nitrogen source.

at pH 8.5 suggested that pH was not an important variable in controlling the total densities of bacteria in the factory fluids. *Acinetobacter* strains and mixed cultures buffered at pH 8.5 (borate buffer) with 0.1% acetate or 5% metal-working fluid as the carbon source grew to the same densities and at the same rates as they did in media with the same carbon sources buffered at pH 7.5.

Discussion

The first point for discussion is to call attention to the presence of *Acinetobacter* species in metal-working fluids. The significance of these bacteria is that under laboratory conditions with metal-working fluid or its components as carbon sources, they grow faster and to higher density than any other species of bacteria isolated from this system. The *Acinetobacter* genus has not yet been extensively studied, having not been defined until 1968[7]. Since 1968, only a few *Acinetobacter* species have been characterized in detail[8,9], and studies of their hydrocarbon metabolism were not begun until about the mid-1970s[5]. Some investigators have suggested that *Acinetobacter* species are important natural degraders of petroleum oils[8]. *Acinetobacter* species have been isolated from river sediments[12], activated sludge[13] and clinical samples[14]. Bacteria that grow on long-chain fatty acids and on various petroleum products have been described since the 1940s[15]. It could therefore be assumed that other investigators have also encountered *Acinetobacter* species in samples of metal-working fluids, but the taxonomic criteria they used for identification did not key onto the genus.

The second point is that even though the *Acinetobacter* species grew very rapidly and to high densities (10^9 CFU/ml) in the laboratory with the metal-working fluids as the sole carbon source, they only accounted for a few percent of the total populations in fluid samples from the factory. The reason the proportion of *Acinetobacter* species was lower in the factory samples than that expected from data on the relative growth rates of pure cultures appears to lie in differences between pure cultures and mixed cultures. The proportions of *Acinetobacter* species in the stationary-phase mixed laboratory cultures (see Table 2) were comparable with those observed for factory samples. The stationary-phase cultures presumably correspond to the populations in a stable well-mixed factory reservoir.

The final point is to suggest that in this particular metal-working fluid, the *Acinetobacter* species are the first bacteria to colonize the fluid and they support the high densities of other species. This suggestion follows from the laboratory experiments with mixed cultures. From a low proportion in the initial inoculum, the *Acinetobacter* species grew to account for more than half of the population in the exponential growth phase. In stationary-phase mixed cultures, the total bacterial density was constant and the *Acinetobacter* species decreased to a few percent, but the density of the other strains, mostly *Pseudomonas* species, remained at over 10^9 CFU/ml even though these latter strains were not capable of growth to this density when cultured as pure strains. The process that supports the growth of the *Pseudomonas* species in the presence of the *Acinetobacter* species is unknown, but it appears that it is not simply a matter of the latter 'conditioning' the fluid. In preliminary experiments, an attempt was made to grow the *Pseudomonas* strains on used metal-working fluid and on fluid in which *Acinetobacter* strains were first grown to

high density and then eliminated by centrifugation and pasteurization. When cultured on this 'conditioned' medium, pure strains of *Pseudomonas* still did not grow to the densities observed when co-cultured with *Acinetobacter* strains.

Conclusions

It should be emphasized that the original intention was to investigate very high levels of bacterial contamination, i.e. 10^9 CFU/ml. The discussion above may not pertain to systems in which the bacterial densities are less than, say, 10^6-10^7 CFU/ml because many species of bacteria are capable of growing to these lower densities with the ingredients of metal-working fluids as carbon sources. It is not intended to suggest that all cases of high density (10^9 CFU/ml) contamination are associated with *Acinetobacter* species. It is suggested as a working hypothesis, however, that cases of extreme contamination are 'triggered'

by one or a small number of closely related species. In the case presented here, it is proposed that the *Acinetobacter* species control the contamination in the sense that they appear to be necessary to support the presence of the other bacteria even though the *Acinetobacter* species are not the most abundant bacteria in the samples from the factory. Presumably, all of the bacterial species are indigenous to the factory environment. The conclusion, then, is that the key to preventing cases of extreme contamination is to identify and understand the metabolic properties of the 'governing' species.

Acknowledgement

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