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Bioaccumulation of Silver by a Multispecies Community of Bacteria

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Abstract. A stable community of bacteria that had unusually high tolerance of soluble silver was isolated from soil by chemostat enrichment. The community consisted of three bacteria: *Pseudomonas maltophilia*, *Staphylococcus aureus* and a coryneform organism. The pseudomonas was primarily responsible for the silver resistance. The tolerance of high silver concentrations, up to 100 mM Ag^+, was greatly reduced when the community was grown in the absence of silver. *Pseudomonas maltophilia* comprised approximately 50% by numbers of the community when grown in chemostats in the presence or absence of Ag^+ but large fluctuations occurred in population sizes of the other two bacteria; the *S. aureus* population was small (less than 1%) in the presence of Ag^+ but comparised a third of the total numbers when Ag^+ was omitted from the medium. Silver-resistant respiration of the silver-adapted community was significant even when it was confronted with high concentrations of Ag^+. In contrast the respiration of the coryneform organism and particularly *S. aureus* was highly sensitive to silver. The inhibition constants for silver-sensitive respiration were 0.78 mM and 0.04 mM for silver acclimatized and non-acclimatized communities respectively.

The community had great capacity for silver bioaccumulation. Maximum concentrations of over 300 mg silver per g dry weight of biomass were recorded at an accumulation rate of 21 mg Ag^+ h^{-1} (g biomass)$^{-1}$. The extent of silver removal from solution was a function of initial concentration of silver; at low external concentrations (ca. 1 mM) all the silver was rapidly removed from solution, at high concentrations (ca. 12 mM) 84% removal occurred in 15 h.

Key words: Silver Bioaccumulation – Bacterial community – *Pseudomonas maltophilia* – Chemostat enrichment isolation.

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Silver is one of the most toxic heavy metals to heterotrophic bacteria (Allbright et al., 1972) and growth may be inhibited by concentrations several orders of magnitude less than comparable inhibitory concentrations of most other metals, including cadmium, zinc and mercury. Indeed, low concentrations of silver salts have long been deployed as antibacterial agents (Foye, 1977). The toxicity of copper may approach that of silver (Allbright et al., 1972) but highly tolerant strains of bacteria are known (Horitsu et al., 1978). Silver toxicity similar to that for heterotrophs has been reported for chemolithotrophic organisms involved in metal leaching (Hoffman and Hendrix, 1976; Norris and Kelly, 1978). The latter authors found that the growth of several iron-oxidising bacteria was inhibited by 10 μM Ag^+ and that toxicity was related to bioaccumulation of the metal; approximately 50 μmol Ag^+ per g dry weight was accumulated by *Thiobacillus ferrooxidans*. The mechanism of silver toxicity may be related to complex formation with sulphydryl groups, interactions with DNA and/or inhibition of electron transport.

It appears that no studies have been made of microorganisms that are tolerant of high concentrations of silver or, apart from the work cited above, of silver bioaccumulation by microorganisms. In contrast, the bioaccumulation of platinum, palladium, gold and mercury is reported to be abnormally high in species of *Pseudomonas* (Chakrabarty, 1976) and continuous microbiological processes have been patented for the recovery of toxic metals such as mercury (Chakrabarty et al., 1975). The object of the research reported in this paper was to attempt the isolation of heterotrophic organisms that were not only highly tolerant of silver but also had the capacity for its bioaccumulation. Microorganisms having such properties might be exploitable in the development of a recovery process for silver from industrial effluents and waste materials. A major source of recyclable silver is photographic waste materials including film and processing reagents.

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We have used continuous-flow enrichment isolation (Veldkamp, 1970) in several projects, including the biodegradation of xenobiotic compounds (Bull and Brown, 1979). This technique enables microorganisms to be selectively isolated under fully defined conditions and, where potentially toxic materials are involved, offers the possibility of adapting populations to progressively higher concentrations. Moreover, enrichment experiments made in chemostats usually result in the selection of mixed cultures of microorganisms and we wished to test the hypothesis that multispecies communities might be more tolerant of toxic metals than monospecies cultures.

Materials and Methods

Growth Medium. A gelatin-mannitol medium having the following composition was used in enrichment isolation and growth experiments ($g\,l^{-1}$): gelatin, 10.0; mannitol, 5.0; yeast extract (Difco), 1.0; KH_2PO_4, 1.5; Na_2HPO_4, 0.5; $MgSO_4 \cdot 7\,H_2O$, 0.15; $CaCl_2 \cdot 6\,H_2O$, 0.01; $FeSO_4 \cdot 7\,H_2O$, 0.01; $MnSO_4 \cdot 4\,H_2O$, 0.002; $ZnSO_4 \cdot 7\,H_2O$, 0.002. The pH was adjusted to 7.0 after autoclaving and silver (as $AgNO_3$) added as a filter-sterilized solution. Agar (Oxoid No. 3, 15 g l^{-1}) was incorporated as required. Partial decomposition of the silver nitrate (presumably to metallic silver) occurred both in liquid and agar media but precipitation of medium constituents was not obvious.

Preliminary Screening for Ag^+-Tolerant Organisms. Samples (10 g) from a range of soil types were shaken with 25 ml sterile water and the suspensions filtered through coarse paper to remove particulate matter. Half of each filtrate was heated to 80° C for 12 min in an attempt to select for proteolytic bacilli; the other half was not heated. Spread plates (0.1 ml) of 10^{-1} to 10^{-3} dilutions were made on gelatin-mannitol agar containing 0, 0.1, 1.0, 10 and 100 mM silver nitrate. After 72 h at 37° C, those plates containing the highest concentrations of silver nitrate and showing growth were twice replicated on to gelatin-mannitol agar and, following incubation, one plate of each pair was developed with mercuric chloride reagent [4 g HgCl in 100 ml 10% (v/v) HCl] to test for gelatinase production.

Chemostat Enrichment of Ag^+-Tolerant Organisms. The preliminary screening tests revealed that proteolytic, silver tolerant organisms were present in several untreated soil suspensions and were resistant to 1.0 mM silver nitrate. Subsequently samples from these soils were used to inoculate chemostats. The equipment used was of the type described by Senior et al. (1976) for the continuous flow enrichment of herbicide-degrading communities. The working volume was 0.50 l; dilution rate 0.05 h^{-1}; temperature 37° C with aeration at 650 ml min^{-1}. pH was not controlled. The culture was put on flow immediately following inoculation of the gelatin-mannitol medium containing 1 mM $AgNO_3$ with filtered soil suspension [5% (v/v)]. Chemostats were monitored regularly for viable organisms using argentiferous and non-argentiferous plating media. The growth-limiting nutrient was not determined.

One enrichment chemostat that ultimately selected a stable population of bacteria (approximately $10^{13}\,l^{-1}$ and comprising three morphologically distinct types) was analysed in detail. A sample of this stabilized, silver tolerant community was used to inoculate another chemostat, identical to the original except that silver was omitted from the medium. These two communities will be referred to as silver-acclimatized and non-acclimatized respectively.

Analyses. Organism dry weights were measured by collecting cells from a 10 ml sample on pre-dried and weighed Millipore filters (0.45 µm pore size) and drying to a constant weight at 110° C. Protein was assayed by the method of Lowry et al. (1951) using gelatin as a standard.

Silver (0.01 – 10 mM) was assayed using an SP 191 atomic absorption spectrophotometer (Pye Unicam Ltd.) with a Corning Activion hollow silver cathode lamp. The presence of gelatin-mannitol medium had no effect on the silver assay.

Silver Bioaccumulation. Samples (50 ml) of cultures were centrifuged, washed and resuspended in gelatin-mannitol medium containing various concentrations of silver and incubated at 37° C with shaking in 250-ml Erlenmeyer flasks. Silver assays were made on the whole organism suspension and on membrane filtrates at various times; concurrently samples were also taken for biomass measurements.

Respirometry. Respirometric measurements were made in a Gilson differential respirometer at 37° C. Bacteria were suspended in gelatin-mannitol medium and their respiration rates recorded in the presence (0.025 – 5.0 mM) or absence of silver ions.

Results

Enrichment Studies. A number of soil samples were screened for the presence of Ag^+ tolerant microorganisms. No Ag^+-tolerant organisms were isolated from soil suspensions that had been heat treated. Accordingly enrichment chemostats were always inoculated with unheated samples.

During the course of a typical chemostat enrichment culture (dilution rate 0.05 h^{-1}) the numbers of Ag^+-tolerant bacteria increased from 186 ml^{-1} to a final concentration of $8.5 \times 10^{13}\,l^{-1}$ within 11 days. Organisms from this chemostat were used to inoculate a second which lacked Ag^+ in the medium and the population size stabilized at $3.8 \times 10^{13}\,l^{-1}$.

When samples from both chemostats were plated on to agar medium containing various concentrations of Ag^+, it was found that bacteria from the non-acclimatized chemostat had a sensitivity to silver comparable to the soil populations from which they had been originally obtained, while those from the acclimatized chemostat were highly tolerant (Fig. 1). Clearly

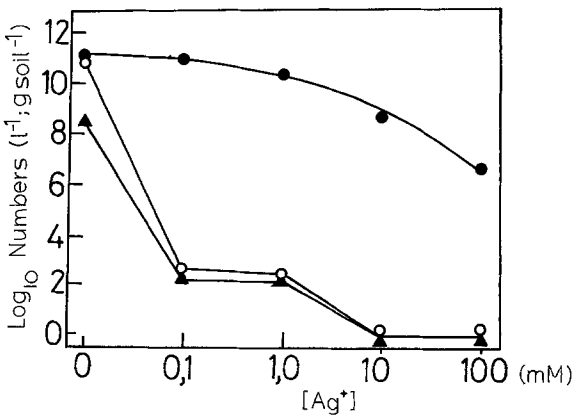

Fig. 1. The response of silver-acclimatized (●) and non-acclimatized (○) chemostat populations to increasing concentrations of Ag^+. The silver sensitivity of the bacterial population present in the original soil sample is also shown (▲)

Table 1. Numbers and proportions of constituent bacteria in silver-acclimatized (1 mM Ag^+) and non-acclimatized chemostat populations

Bacterium	Acclimatized population		Non-acclimatized population	
	Viable count[a]	%	Viable count[a]	%
Staphylococcus aureus	7.0×10^{11}	0.8	1.3×10^{13}	33.0
Pseudomonas maltophilia	4.0×10^{13}	46.7	2.1×10^{13}	54.1
Coryneform organism	4.5×10^{13}	52.5	5.0×10^{12}	12.9
Total	8.57×10^{13}		3.9×10^{13}	

Dilution rate, $D = 0.05\,h^{-1}$

[a] Numbers per litre of culture

silver tolerance was lost from the population when the selection pressure of silver in the medium was removed and this occurred within approximately 80 h of removing the silver.

Community Analysis. The stable, silver-tolerant community was found to consist of three distinct bacterial types: a Gram-positive coccus, a Gram-positive rod and a Gram-negative, non-fermentative rod. These organisms were readily distinguished on agar plates incubated for 72 h on the basis of colony size and pigmentation. Subsequently the identity of these bacteria was determinded at the Torry Research Station (Aberdeen, Scotland) as follows: (i) a strain of *Staphylococcus aureus* (coagulase positive and antibiotic sensitive), (ii) a coryneform, and (iii) a strain of *Pseudomonas maltophilia* having a growth factor requirement satisfied by L-methonine. The proportions of these three bacteria varied in the Ag^+-acclimatized and non-acclimatized chemostat populations (Table 1). *Pseudomonas maltophilia* comprised approximately half of the population in both cultures but wide variations were observed in the Gram-positive species. *Staphylococcus aureus* was a minor component of the Ag^+-acclimatized community but increased dramatically in the absence of silver ions, whereas the coryneform organism became the minor component in the non-acclimatized community.

The capacity to utilize gelatin was reduced when the community was exposed to Ag^+. Thus, the non-acclimatized community utilized gelatin at a rate [75.6 mg (g biomass)$^{-1}$ h^{-1}] more than double that found in the silver-acclimatized culture (32.6 mg g^{-1} h^{-1}).

The silver-acclimatized community was able to remove 75.8 ± 5.6% of the silver supplied to a chemostat population when the feed concentration was 1 mM Ag^+. This capacity to strip silver from solution was maintained during a prolonged period of continuous culture (> 2000 h) and was equivalent to a removal rate of 38 nmol ml^{-1} h^{-1}. Recoveries of silver in the culture effluent approximated to 100% indicating that there were negligible losses due to adsorption onto equipment surfaces.

Effect of Ag^+ on Respiration. Respiratory activities of the acclimatized and non-acclimatized chemostat communities and of monocultures of the constituent bacteria were measured in the presence of varying concentrations of silver ion. The monocultures were derived from the silver-acclimatized chemostat community by separation on argentiferous agar medium and a subsequent single batch culture in non-argentiferous liquid medium. The silver-acclimatized community had considerably greater respiratory resistance to Ag^+ than the non-acclimatized community and retained 10% of its control rate even when challenged with 5 mM Ag^+ (Table 2). Silver-resistant respiration of the acclimatized community closely paralleled that of *P. maltophilia* when the latter was tested in monoculture. In contrast *S. aureus* was particularly sensitive to Ag^+ and its q_{O_2} was reduced to 6% of the control value by 0.05 mM Ag^+ (Table 2).

The differential susceptibility of communities and individual species was also evident from values of inhibition constants for Ag^+ (Table 3). These data indicated that respiration of the acclimatized community was approximately 25 times more resistant to Ag^+ than that of the non-acclimatized community while the community as a whole had a higher K_i than that of the most resistant component, namely *P. maltophilia.*

Silver Bioaccumulation. A series of batch culture experiments was made to measure the rates and extent of silver bioaccumulation by the two communities and by individual species at several initial concentrations of silver (Figs. 2, 3 and 4). It is clear that the acclimatized community had the greatest affinity for silver irrespective of the initial metal concentration; *P. maltophilia* also had high silver bioaccumulation capacity when the initial loading was of the order of 1 mM. In contrast silver bioaccumulation by the unacclimatized community and the coryneform organism was much less

Table 2. Respiration rates in gelatin-mannitol medium of silver-acclimatized and non-acclimatized chemostat communities and their constituent bacteria in the presence of silver

Silver concentration (mM)	Respiration rate (ml O_2 (g biomass)$^{-1}$ h^{-1}				
	Acclimatized community	Non-acclimatized community	*Staphylococcus aureus*	Coryneform species	*Pseudomonas maltophilia*
0	78.9	168.4	265.0	36.5	103.1
0.025	–	–	22.9	19.7	61.5
0.05	45.1	17.1	17.1	14.1	44.7
0.50	34.0	2.7	8.5	8.9	34.2
1.25	27.5	1.1	2.1	5.9	39.3
2.50	12.8	0	0	2.0	14.8
5.00	7.9	0	0	0	7.2

The individual species were isolated from the silver-acclimatized chemostat and grown as monocultures in shake flasks on gelatin-mannitol medium in the presence *(P. maltophilia)* or in the absence *(S. aureus;* coryneform) of 1 mM Ag^+

Table 3. Inhibition constants determined for silver sensitive respiration

	Acclimatized community	Non-acclimatized community	*Staphylococcus aureus*	Coryneform species	*Pseudomonas maltophilia*
K_i (mM)	0.78	0.03	0.04	0.18	0.55

All values obtained from linear regression analysis on plots of $(q_{O_2})^{-1}$ against silver concentration (Dixon and Webb, 1964). Probabilities are > 99.9% in all cases

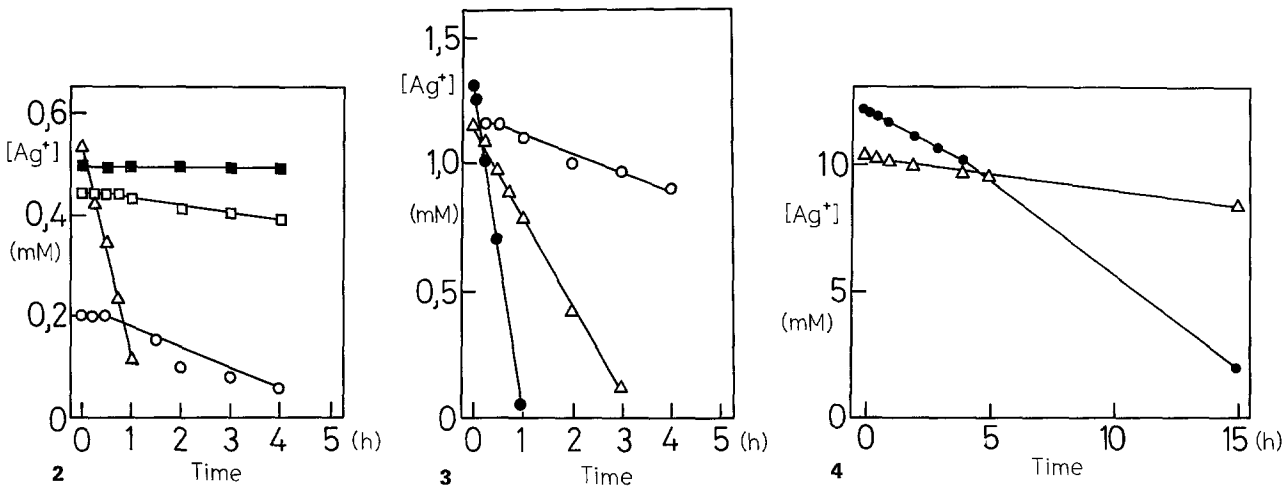

Fig. 2. Silver bioaccumulation kinetics: initial silver concentration in the medium less than 0.6 mM. *Pseudomonas maltophilia* (△), *Staphylococcus aureus* (■), coryneform organism (□) and non-acclimatized community (○)

Fig. 3. Silver bioaccumulation kinetics: initial silver concentrations in the medium approximately 1 mM. *Pseudomonas maltophilia* (△), acclimatized community (●) and non-acclimatized community (○)

Fig. 4. Silver bioaccumulation kinetics: initial silver concentrations in the medium approximately 10 mM. *Pseudomonas maltophilia* (△) and acclimatized community (●)

and characterised by a lag of 30–45 min before silver was removed from the medium. *Staphylococcus aureus* was unable to bioaccumulate silver even from media containing low concentrations of silver (ca. 0.5 mM).

From these experiments, specific rates of silver bioaccumulation as a function of external concentration were calculated for the acclimatized community and *P. maltophilia*. The community showed consistently higher rates of bioaccumulation and its superiority over *P. maltophilia* became more pronounced with increasing silver concentration. At 1 mM Ag^+ the bioaccumulation rate of the community (38 mmol

$g^{-1}\,h^{-1}$) was 20% higher than the *Pseudomonas* rate; at 5 and 10 mM Ag^+ the community bioaccumulation rates decreased to 25 and 20 mmol $g^{-1}\,h^{-1}$ respectively and these were 43% and 82% higher than the comparable *Pseudomonas* rates.

Maximum concentrations of accumulated silver after 15 h incubation were 2.925 mmol (g biomass)$^{-1}$ [316 mg (g biomass)$^{-1}$] for the acclimatized community and 1.687 mmol (g biomass)$^{-1}$ [182 mg (g biomass)$^{-1}$] for *P. maltophilia*. These latter values relate to the highest external concentrations of silver examined (12.15 mM and 10.36 mM respectively) and represent 84% and 19% removal of silver from solution, respectively. At lower external silver concentrations (approximately 1 mM), both the acclimatized community and *P. maltophilia* removed 100% silver from solution very rapidly.

Discussion

Both the tolerance of and the accumulation of silver by the acclimatized community and *Pseudomonas maltophilia* are strikingly high and novel. They are approximately 60 and 35 times respectively greater than that reported for *Thiobacillus ferrooxidans* by Norris and Kelly (1978). We have not determined at what site(s) in the bacteria silver is deposited but the extent of bioaccumulation suggests that the process is not simply surface adsorption. The fact that the rates of silver bioaccumulation by the acclimatized community and *P. maltophilia* were inhibited as the external concentration of Ag^+ increased is noteworthy. Thus, silver accumulation may be respiration dependent while respiration itself is sensitive to silver (Table 2): consequently the extent of bioaccumulation would be a compromise between these two effects. The mechanism of silver tolerance operating in *P. maltophilia* also remains to be investigated but an enzymatic reduction of Ag^+ to Ag°, analogous to the tolerance to mercury described by Furukawa and Tonomura (1972) is presumably feasible. Whatever the basis of this high tolerance of silver, the performance of the acclimatized community is significantly greater than that of *P. maltophilia* alone, irrespective of whether respiration, bioaccumulation or tolerance per se is used as a basis for comparison. The value of continuous-flow enrichment isolation and the potential of mixed cultures in physiological research is again emphasized by this study. The interactions that occur between species even in this simple, 3-membered community are likely to be complex and await resolution. Among the attributes that may be significant in maintaining the integrity of the community are: the high gelatinase activity of *Staphylococcus aureus;* the high sequestration capacity of *P. maltophilia;* and cross-feeding between species. In the latter context, it is known that the *P. maltophilia* has a growth factor requirement that is satisfied by L-methionine. The coryneform organism has a low affinity for silver but, nevertheless, is the dominant species when the community is grown in an argentiferous medium: its greater tolerance of silver than *S. aureus* may make it a critical provider of growth factor(s) for *P. maltophilia* in such environments.

Pseudomonas maltophilia is primarily responsible for silver uptake by the community but in silver-free medium this capacity is lost or drastically reduced despite the fact that it is the dominant species. Such an observation provides some circumstantial evidence for the presence of a plasmid that specifies the silver tolerance mechanism. Plasmid-borne resistance factors for heavy metals such as mercury resistance in *Staphylococcus aureus*, has been reported (Summers et al., 1975). The mercury-reducing system encoded on the *S. aureus* plasmid can also use Ag^+ as a substrate but does not confer resistance to silver. Summers et al. proposed that Ag^+ was not an effective inducer of the mercury-reducing system in this species.

The controlled removal or recovery of toxic heavy metals by microorganisms operating under appropriate process conditions has been advocated by several workers (e.g. Chakrabarty et al., 1975; Iverson and Brinckman, 1978). The present results reveal that this option clearly exists for extremely toxic metals like silver. A continuous process would be most efficient for silver recovery. The chemostat experiments reported above were operated such that the silver removal rate was 0.98 mg (g biomass)$^{-1}$ h^{-1} (dilution rate = 0.05 h^{-1} and an input medium containing 1 mM Ag^+). However, results from the batch accumulation experiments (for example, at an initial Ag^+ concentration of 12.15 mM the removal rate was 21 mg (g biomass)$^{-1}$ h^{-1} suggested that these chemostats were substantially underloaded with silver. Consequently it is not unreasonable to project that the deployment of multistage continuous-flow reactors, with or without partial organism recycle, would enable total recovery of silver from concentrated solutions. This possibility is currently being investigated.

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