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Metal Resistance in *Acidocella* Strains and Plasmid-Mediated Transfer of This Characteristic to *Acidiphilium multivorum* and *Escherichia coli*

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Acidophilic heterotrophic strain GS19h of the genus *Acidocella* exhibited extremely high resistance to $CdSO_4$ and $ZnSO_4$, with a MIC of 1 M for each. The respective MICs for an *Acidocella aminolytica* strain were 400 and 600 mM. The MICs of $NiSO_4$ for the above strains were 200 and 175 mM, respectively. These strains were also resistant to $CuSO_4$, the MICs being 20 and 40 mM, respectively. An *Acidocella facilis* strain showed resistance only to $ZnSO_4$, with a MIC of 150 mM. The metal salts, in general, extended the lag period, log period, and generation time, with decreases in growth rate and optimum growth. *A. aminolytica* and strain GS19h each contain more than one plasmid, while *A. facilis* contains none. After transformation by electroporation with the plasmid preparation from strain GS19h, an *Acidiphilium multivorum* strain became highly resistant to cadmium and zinc, and the plasmid profile of the transformed cells was found to differ from that of the original *Acidiphilium multivorum* strain. *Escherichia coli* HB101 and DH5α also exhibited more resistance to these metals, especially zinc, after transformation with the total plasmid preparation of strain GS19h or a 24.0-MDa plasmid of the same strain, although no plasmid was detected in the transformed cells. Thus, the results derived mainly through genetic experiments demonstrate for the first time the plasmid-mediated transfer of metal resistance for an acidophilic bacterium.

In ore-leaching environments, resistance to several metals is observed in a wide variety of bacteria, including the well-studied bacterium *Thiobacillus ferrooxidans* (31, 32) and related acidophiles (13, 14). This property is considered the most suitable phenotypic trait for selection of genetically engineered mining microbes (24, 25, 28). Genetic inheritance of metal resistance is often located on plasmids (29, 32), but for acidophiles such a list awaits generation. Because of the importance of plasmid-borne metal resistance in genetic manipulation of leaching microbes (24, 25) and due to the very low to nonexistent level of expression of this characteristic in heterologous systems (18), endogenous plasmids carrying genes for this phenotypic trait in *T. ferrooxidans* or other acidophiles may find wide application in the development of bioleaching operations (3, 28). Since many acidophilic bacterial strains contain plasmids (8, 15, 16, 21), there is a high probability that a few of them harbor metal resistance-conferring plasmids. In the case of *T. ferrooxidans*, several examples of a circumstantial relationship between the inheritance of a plasmid and a metal-resistant phenotype have been reported (17, 22, 23, 33), but no direct evidence in favor of plasmid-borne metal resistance has been established. In this species, resistance markers for arsenic (11), copper (19), mercury (27), and zinc (11) have been found or have been suggested to be chromosomally located.

Similarly, in the case of highly metal-resistant acidophilic heterotrophic strains of the genus *Acidiphilium* (14), plasmid-mediated resistance to metals has not yet been demonstrated. Recently a new group of acidophilic heterotrophs of acidic mineral environments has been described (10); one strain of this genus, *Acidocella* sp. strain GS19h, was found to be highly zinc resistant and to contain stable plasmids (1, 13). To evaluate it and two other strains of the *Acidocella* genus (10) as potential sources of metal resistance marker genes in developing genetic technology for acidophiles, the metal resistance property of these strains, vis-à-vis its inheritance on their plasmids, was studied. The plasmid-mediated transfer of this property to an acidophilic bacterium and *Escherichia coli*, as presented in this work, is direct evidence, for the first time, of plasmid inheritance of metal resistance in an acidophilic strain.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Several *E. coli* strains and acidophilic heterotrophs (Table 1) were used in this study. *E. coli* strains were cultured in Luria-Bertani (LB) broth or on LB agar (26) at 37°C. The metal-containing LB agar plates were used for selection of *E. coli* transformants and for determination of the MICs of metal salts for them. These were prepared by adding the requisite volume of a 1 M salt solution to LB broth followed by adjustment of the pH to 7.2 to 7.3 with 5 N NaOH solution, addition of agar (1.5%, wt/vol), and sterilization. Acidophilic heterotrophs were grown at 30°C in MGY medium, of the following composition (in grams per liter) (14): $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 0.25; $MgSO_4 \cdot 7H_2O$, 0.25; KCl, 0.1; glucose, 1.0; and yeast extract, 0.1. The pH was adjusted to 3 with 1 N H_2SO_4. Selection of transformants of the acidophilic heterotrophs was made on MGY-agarose (0.6%, wt/vol), which was prepared by adding an equal volume of sterile 2× MGY medium containing a metal salt (pH 3) to sterile 1.2% (wt/vol) agarose just before plating. MICs of the metal salts for these strains and their transformants were determined in liquid medium.

Determination of the metal resistance spectrum and evaluation of growth parameters. The MICs were determined in triplicate by allowing the *Acidocella* strains to grow for 7 days in metal salt-containing MGY medium (pH 3) at 30°C on a rotary shaker. Growth of bacteria was monitored by measuring the optical density (OD) at 540 nm, and growth parameters were calculated from the curve of $\ln (OD_t/OD_0)$ versus time, as described previously (14). Concentrations of metal salts for determining the growth parameters were such that the growth attained stationary phase within 4 days.

Preparation of plasmids. Plasmids were isolated according to the method of Birnboim and Doly (2), or by a method applied for acidophilic heterotrophs (21), and purified by CsCl-ethidium bromide gradient centrifugation as required (26).

In practice, for small-scale plasmid preparation from acidophilic heterotrophs, saline-washed cells from a 20-ml culture were incubated with 100 μl of solution I (26) containing 10 mg of lysozyme per ml for 10 min at room temperature. After addition of 200 μl of solution II (26), the mixture was kept on ice for 5 min followed by addition of 150 μl of solution III (26) and incubation on ice for 20 min. The clear supernatant obtained after centrifugation was extracted with an

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristic(s)	Reference(s) or source
E. coli strains		
HB101	*sup hsd recA*	26
DH5α	*sup hsd recA*	26
S17-1(pSUP106)	*rec hsd* Tra^+ (Cm^r Tc^r)	20
V517	Wild type	12
Acidiphilium multivoram	Wild type	34
Acidocella sp. strains	Wild type	1, 10
Plasmids		
From acidophilic strains		This study
From *E. coli* V517	Molecular size markers	12
pBR322	2.84 MDa, Ap^r Tc^r	Sigma
pSUP106	6.5 MDa, Cm^r Tc^r Tra^-	20
RP4	37.2 MDa, Km^r Tc^r	5
POT3	29.7 MDa, Tc^r	7

equal volume of phenol-chloroform (1:1, vol/vol) and chloroform, used once each. Precipitation of plasmid DNA was carried out with isopropanol. The pellet was washed with 70% ethanol, dried, and dissolved in 30 to 40 μl of Tris-EDTA buffer or water. Large-scale preparation of plasmids was also done by this method.

In another method, washed cells from a 20-ml culture were suspended in 200 μl of Tris–acetate (40 mM)–EDTA (2 mM), pH 7.9, and were lysed with 400 μl of 3% (wt/vol) sodium dodecyl sulfate–50 mM Tris (pH 12.6), followed by incubation at 62 to 64°C for 20 to 25 min. The mixture was then treated with phenol-chloroform and centrifuged, and the aqueous layer was extracted with chloroform. Thereafter, 200 μl of 3 M sodium acetate was added to the aqueous phase, and plasmid DNA was precipitated and purified as described above.

Electrophoresis and elution of plasmids from gels. Plasmids in gel (0.5 to 0.8% [wt/vol] agarose) electrophoretograms were detected after ethidium bromide (0.5 μg/ml) staining, as usual (26). The molecular sizes of different plasmids were determined by comparison with standard plasmids of known molecular sizes (5, 7, 12). Elution and purification of plasmids from the agarose gels were performed as described previously (26).

Electrotransformation of acidophilic heterotrophs. *Acidocella facilis* and *Acidiphilium multivorum* strains were grown at 37°C to late log phase in MAS medium (6), of the following composition: 1 mM $(NH_4)_2SO_4$, 2 mM KCl, 0.86 mM K_2HPO_4, 10 mM $MgSO_4$, 6.6 mM $CaCl_2$, 2.6 mM $FeSO_4$, 0.01% (wt/vol) yeast extract, and 0.1% (vol/vol) glycerol (pH 3.0). Cells from a 1-ml culture were harvested, washed twice with 200 μl of 20% (vol/vol) glycerol, and suspended in 100 μl of the same. Twenty microliters of cell suspension was mixed with 200 μg of plasmid DNA from *Acidocella* sp. strain GS19h, and a 15-kV/cm pulse was passed for 10 ms in a Cell-Porator instrument (BRL Life Technologies, Inc.). In the control sets, instead of DNA solution, an equal volume of water was added. The cells were then mixed with 379 μl of MAS medium containing either 50 mM $ZnSO_4$ or 30 mM $CdSO_4$ and stored with shaking at 30°C for 40 to 42 h. Selection of the transformed *Acidiphilium multivorum* cells was done on MGY-agarose plates containing 100 mM $ZnSO_4$ or 40 mM $CdSO_4$. Since *A. facilis* exhibited significant resistance to $ZnSO_4$, selection of transformed *A. facilis* cells was made on MGY-agarose plates containing 8 mM $NiSO_4$ or 2 mM $CdSO_4$.

Transformation of *E. coli* strains. CsCl gradient-purified plasmid DNA of *Acidocella* sp. strain GS19h was used for transformation of $CaCl_2$-treated cells of *E. coli* strains (26), while no DNA was added to these cells in the control sets. To check whether competency of *E. coli* cells was attained, another transformation experiment was conducted with pBR322 DNA. Selection of transformed *E. coli* cells was made on LB agar containing either $ZnSO_4$ (12 and 16 mM) or $CdSO_4$ (8 and 12 mM) in the case of plasmid DNA of *Acidocella* sp. strain GS19h, while for pBR322 tetracycline (30 μg/ml)-containing LB agar was used.

Southern hybridization. The plasmid DNA preparation from *Acidocella* sp. strain GS19h was subjected to random primer labelling with [^{32}P]dATP. Hybridization of this probe with plasmid DNAs from transformed and wild-type *Acidiphilium multivorum* cells, and other sources blotted on a nylon filter, was carried out as described by Sambrook et al. (26).

RESULTS AND DISCUSSION

The genus *Acidocella* contains only two species (10). In this study, one strain from each of these species and an unclassified strain (1) were used. These strains showed variable degrees of resistance to one or more of four metals: cadmium, copper, nickel, and zinc (Table 2). Strain GS19h exhibited extreme resistance to both Cd^{2+} and Zn^{2+} and very high resistance to Ni^{2+}. *Acidocella aminolytica* also showed high resistance to these metals. Higher resistance to Cu^{2+} was observed in this strain than in other acidophilic heterotrophs (14). *A. facilis* showed some resistance only to Zn^{2+} and was unable to grow in the presence of other metals at low concentrations. Thus, the *Acidocella* strains, in general, possess some tolerance to Zn^{2+} in comparison with other metal ions. Moreover, among the prokaryotes, *Acidocella* sp. strain GS19h exhibited the highest resistance reported thus far to both Cd^{2+} and Zn^{2+}. Some growth parameters of these strains at subinhibitory concentrations of resistant metals are presented in Table 3. It may be noted that in strain GS19h, the metals Cd, Cu, and Ni extend the lag period (t_λ) to a great extent, with extension of the total growth period (t_α) and generation time (T). Optimum growth of this strain was up to 70 to 80% of that of the control in the presence of these metals. In the case of *A. aminolytica*, optimum growth in the presence of zinc was sufficiently low compared to the control and other experimental sets containing metals. In general, metals extended the lag period, log period, and generation time, with decreases in growth rate and total growth.

TABLE 2. MICs of metals for *Acidocella* strains in MGY medium

Bacteria	MIC (mM)[a]			
	$3CdSO_4 \cdot 8H_2O$	$CuSO_4 \cdot 5H_2O$	$NiSO_4 \cdot 6H_2O$	$ZnSO_4 \cdot 7H_2O$
Acidocella sp. strain GS19h	1,000 (700)	20 (15)	200 (150)	1,000 (900)
A. aminolytica	400 (200)	40 (30)	175 (150)	600 (500)
A. facilis	1[b]	1[b]	3 (1)	150 (100)

[a] Numbers in parentheses indicate the next lower concentration of the metal salt at which growth was observed.
[b] Lower concentrations were not tested.

Metal resistance is inherited by plasmids in many bacteria (29, 32). Therefore, the plasmid profiles of these strains were studied to find the genetic basis of metal resistance in *Acidocella* strains. *A. aminolytica* and strain GS19h each possessed more than one plasmid, while the *A. facilis* strain had none (Fig. 1). The single DNA band detected on the gel electrophoretogram in the case of *A. facilis* was found to be of chromosomal origin after restriction digestion. The approximate

TABLE 3. Effects of metal salts on growth parameters of *Acidocella* strains[a]

Strain	Metal (concn [mM])	t_λ (h)	t_α (h)	a	K (h^{-1})	T (h)
Acidocella sp. strain GS19h	Control	3.2	16.5	2.3	0.166	4.2
	Cd (200)	14.5	68.0	1.8	0.033	20.0
	Cu (5)	25.0	52.2	1.7	0.063	11.0
	Ni (50)	36.5	87.5	1.7	0.033	20.8
	Zn (350)	5.0	28.8	1.6	0.064	10.8
A. facilis	Control	3.5	19.6	4.3	0.287	2.4
	Zn (50)	6.6	35.2	3.3	0.113	6.1
A. aminolytica	Control	4.4	17.5	3.2	0.225	3.1
	Cd (100)	5.5	34.0	2.4	0.085	8.2
	Cu (15)	6.4	47.0	2.5	0.059	11.7
	Ni (50)	8.5	49.5	2.4	0.058	11.8
	Zn (175)	7.3	29.0	1.5	0.068	10.2

[a] Values of t_λ (lag period), t_α (end of exponential phase), and a (asymptotic level) were determined directly from growth curves. K (specific growth rate) and T (generation time) were obtained from the curves as described previously (14).

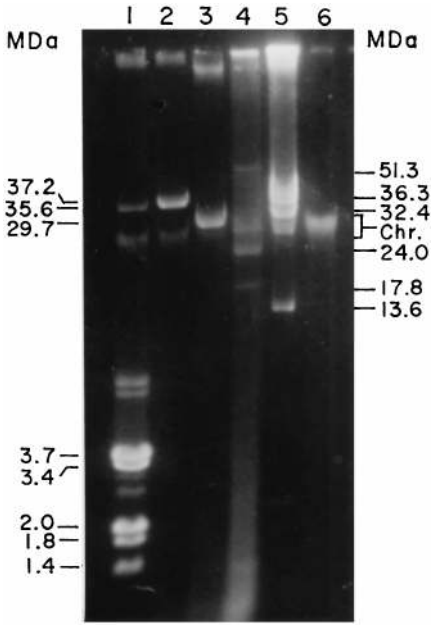

FIG. 1. Plasmid analysis of *Acidocella* strains in an agarose gel (0.5%, wt/vol). Lane 4, *Acidocella* sp. strain GS19h; lane 5, *A. aminolytica*; lane 6, *A. facilis*. Molecular size markers: lane 1, *E. coli* V517; lane 2, *E. coli* C600(RP4); lane 3, *E. coli* C600(POT3). Chr., chromosomal DNA.

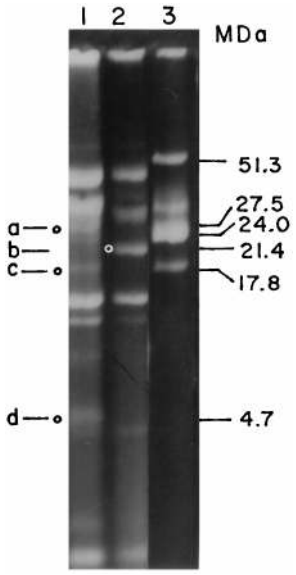

FIG. 2. Agarose gel (0.7%, wt/vol) electrophoretogram of plasmid DNA samples. Lane 1, *Acidiphilium multivorum*; lane 2, *Acidiphilium multivorum* transformed with GS19h plasmid DNA; lane 3, *Acidocella* sp. strain GS19h. Bands of interest are indicated on the left side of the gel.

sizes of the plasmids in *A. aminolytica* were 36.3, 32.4, and 13.6 MDa, and those in strain GS19h were 51.3, 24.0, and 17.8 MDa.

Since the highly metal-resistant strains of *Acidocella* contain plasmids, there is still the possibility that these elements harbor the related genes. Circumstantial evidence in support of this possibility was the observation that the MICs of $CdSO_4$ and $ZnSO_4$ decreased 40-fold, from 1 M for strain GS19h (wild type) to 25 mM for a plasmidless derivative of the same strain. Metal resistance determinants are expressed to some extent better in homologous systems (18). Therefore, attempts were made to introduce the plasmids from *A. aminolytica* and strain GS19h by electroporation into *A. facilis*, a comparatively more metal-sensitive and plasmidless strain. However, no metal-resistant colonies of *A. facilis* were detected after repeated attempts at electrotransformation.

Thereafter, a closely related heterologous system, the acidophilic heterotroph *Acidiphilium multivorum*, was subjected to transformation with plasmids of *A. aminolytica* and strain GS19h by electroporation. Compared to the metal-resistant plasmid hosts, the MICs for the recipient *Acidiphilium multivorum* strain (Tables 2 and 4) were only 20 mM for $CdSO_4$ and 40 mM for $ZnSO_4$ (14). However, after transformation with plasmids from metal-resistant *Acidocella* strains, cadmium- and zinc-resistant colonies appeared on selection plates only in the case of the plasmid DNA preparation from strain GS19h with an efficiency of 2.2×10^3 transformants per μg of plasmid DNA. The MICs of Cd^{2+} and Zn^{2+} for the transformed cells were much higher than those for the parent (Table 4), indicating plasmid-mediated development of metal resistance in this strain. Since no colony appeared on the metal-containing selection plates in the control experiments, it was concluded that the metal-resistant clones were true transformants and not spontaneous mutants.

TABLE 4. MICs of metal salts for the transformants

Bacterial strains	MIC (mM)[a]		
	$3CdSO_4 \cdot 8H_2O$	$CuSO_4 \cdot 5H_2O$	$ZnSO_4 \cdot 7H_2O$
Transformed *Acidiphilium multivorum*	400 (20)		800 (40)
Transformed *E. coli* DH5α	8 (1)	32 (10)	64 (10)
Transformed *E. coli* HB101	8 (1)	32 (8)	48 (8)

[a] Numbers in parentheses indicate the concentrations at which untransformed cells were unable to grow.

The plasmid profiles of the transformed derivatives of *Acidiphilium multivorum* differed from the original profile of the strain with the appearance of a new plasmid band, band b, of ca. 21.4 MDa in the gel electrophoretogram (Fig. 2, lane 2). This new band did not match (on the basis of molecular size) either of the plasmid bands of strain GS19h. Moreover, three plasmid bands, a, c, and d (Fig. 2, lane 1) were not detected in the transformed *Acidiphilium multivorum* cells. Observations of a similar nature were reported earlier when plasmids RP4 and pVK101 were conjugally transferred from *E. coli* into *Acidiphilium cryptum* DSM2389 containing five indigenous plasmids (21). It was observed, however, that the change in plasmid profile of the *Acidiphilium multivorum* transformants in comparison with the untransformed cells was not stable; in the absence of metal stress the plasmid profile of the transformant changed to that of the untransformed strain with concomitant loss of the increased level of resistance to cadmium and zinc. The absence of bands a, c, and d (Fig. 2) in the plasmid profile of transformed *Acidiphilium multivorum* when it was grown in the metal-containing MGY medium was probably due to a drastic decrease in their copy number, as was reported in the case of *T. ferrooxidans* grown in the presence of copper (19). It should be noted that no change in the plasmid pattern of the wild-type *Acidiphilium multivorum* strain took place when it was grown in MGY medium containing metal salts.

To find out whether the new plasmid band in transformed *Acidiphilium multivorum* came from the donor strain, GS19h,

hybridization experiments were performed. However, no conclusion could be drawn from these experiments, since the original plasmids of *Acidiphilium multivorum* hybridized with those of strain GS19h. Probably, plasmids of these strains contained stretches of homologous regions or similar structural motifs related to some of their common physiological functions. It should be mentioned that *T. ferrooxidans* strains isolated from different sites contain plasmids which have highly homologous regions (3, 28).

E. coli is often the organism of choice for the cloning of foreign genes and study of their characteristics. Therefore, to get more information on the metal-resistant plasmids of the *Acidocella* strains, development of metal resistance in *E. coli* through these plasmids was next investigated. It was, however, reported that the metal-resistant genes of *Alcaligenes eutrophus* escaped expression in *E. coli* (30). In spite of this, attempts were made to transform competent *E. coli* strains with the plasmids of *Acidocella* strains because of the very high level of metal resistance in these acidophiles compared to *Alcaligenes eutrophus* and expression of this characteristic in a similar but heterologous *Acidiphilium* strain by the GS19h plasmid(s). It was expected that even low-level expression of metal resistance genes of *Acidocella* strains in *E. coli* might help selection of transformed *E. coli* cells after their incorporation of a metal-resistant plasmid. Since conjugation is a very efficient method of gene transfer, this technique was attempted first to obtain metal-resistant derivatives of *E. coli* HB101 and DH5α. Although the majority of the *Acidocella* plasmids are of high molecular weights, no metal-resistant colony was isolated from either direct or triparental mating experiments using *E. coli* S17-1 containing pSUP106. The results suggested that even if metal resistance genes are located in any of the plasmids of *A. aminolytica* or strain GS19h, the plasmid is not of a conjugative type, has a limited host range, or remains in a repressed state. Nonexpression of metal resistance genes in *E. coli* (30) and failure of *Acidocella* strains to mate with *E. coli* are other possibilities.

Transformation of *E. coli* strains, however, yielded metal-resistant colonies on $ZnSO_4$ (12 mM)-containing LB medium with the plasmid preparation from strain GS19h only. Transformation efficiencies for strains HB101 and DH5α were determined to be 2.9×10^2 and 2.4×10^3 per μg of plasmid DNA, respectively; however, with positive control plasmid pBR322 DNA, the transformation efficiencies were as high as 5.5×10^6 and 9.8×10^6, respectively. Under identical conditions, no colony was detected in the control set containing $CaCl_2$-treated competent cells and no plasmid. The transformed colonies retained the Zn resistance characteristic even after repeated transfer on Zn^{2+}-free liquid or agar medium. Some cross-resistance to cadmium and copper was also observed in these colonies, which were able to tolerate as much as 32 mM Zn^{2+} on direct transfer from a Zn^{2+}-free culture (Table 4).

To determine which of the plasmids of strain GS19h conferred metal resistance in *E. coli* strains, plasmid bands of ca. 17.8 and 24.0 MDa were eluted from the agarose gel, and transformation of *E. coli* HB101 and DH5α was carried out with these purified preparations. While no metal-resistant colony was detected in the case of the smallest plasmid, of 17.8 MDa, resistant colonies appeared on $ZnSO_4$-containing LB agar medium after transformation with the ca. 24.0-MDa plasmid. This result strongly suggested inheritance of Zn resistance in this plasmid of strain GS19h.

Interestingly, however, no stable plasmid band was detected in the agarose gel electrophoretogram of the preparations from these metal-resistant transformed clonal derivatives in either case. Although a faint plasmid band at the level of the ca. 24.0-MDa plasmid band of strain GS19h was detected initially with some metal-resistant derivatives, after repeated culturing this band became no longer visible. Also, no separate plasmid band was detected after CsCl density gradient centrifugation of the clear lysate of the transformed cells, indicating the absence of any plasmid in the transformed *E. coli* cells. The result is reminiscent of a previous observation (9), in which a chimeric plasmid band of 8.8 kb gradually disappeared in transformed *A. facilis* cells although they retained the donor's phenotypic property. In all probability, the ca. 24.0-MDa plasmid of strain GS19h, or a part of it, became incorporated into the chromosome of transformed *E. coli* cells, imparting to it the metal resistance characteristic through a transposon-like element. It should be mentioned that a broad-host-range plasmid of *T. ferrooxidans* contains a Tn*21*-like transposon which has a truncated mercury resistance operon (4). Similarly, a transposon with a complete metal resistance operon may be present in other acidophilic bacteria. The results obtained with the plasmids of *Acidocella* sp. strain GS19h predict such a probability, i.e., the presence of a metal resistance-conferring transposon in at least one of the plasmid DNA molecules of strain GS19h.

In conclusion, this work demonstrates for the first time that the plasmids of acidophilic heterotrophs are not cryptic in nature but impart metal resistance in host cells.

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