Microbial Formation of Oxalate Films on Monument Surfaces: Bioprotection or Biodeterioration?

MARIA PIA DI BONAVENTURA
MADDALENA DEL GALLO
PAOLA CACCHIO
CLAUDIA ERCOLE
ALDO LEPIDI

Department of Basic and Applied Biology
Microbiology Laboratory
University of L'Aquila
Coppito, L'Aquila, Italy

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Oxalate films observed on stone monument surfaces deserve greater interest because of their possible role in protecting against deterioration. Their origin remains controversial. We present here the results of research conducted on production of oxalic acid and other organic acids by bacterial communities isolated from two monuments. Both communities were developed in vitro, and oxalate production was evaluated in a context of global metabolic activities that could eventually lead to protection or to degradation of the surface itself. HPLC analyses of organic acids production revealed that all mixed cultures produced oxalic acid but in different amounts. Besides oxalic acid, other organic acids are released that can solubilize stone calcium carbonate and have a deteriorating activity. Calcium carbonate solubilization, evaluated both by mixed cultures and isolated strains, was stronger with mixed cultures than with single strains. Our data show that oxalate production is promoted by the bacterial communities inhabiting the monument surface: Oxalate, being a minor representative among the organic acids released by the microbe cultures in a relatively short-term analysis, could form insoluble calcium salts that progressively accumulate.

Keywords biodeterioration, bioprotection, microbial oxalate production, oxalate biofilms

Calcium oxalate films observed on stone monument surfaces deserve greater interest, particularly because of the possibility that they could eventually exert a positive role in protecting the monument itself from deterioration. When these "patinas" are intact, the monument surface is generally well preserved. On the contrary, when they are weathered, monument surface degradation is frequently observed. At present, the trend with regard to restoration of ancient buildings is to preserve these patinas (Fassina 1989).

Their origin remains controversial: Different hypotheses consider this coating either an "artificial product" or a "natural one." An "artificial product" in the case of surface treatments is when the presence of oxalic acid can be ascribed either to the microbial or spontaneous transformation of organic substances utilized to preserve the monument or, directly, to the utilization of oxalic acid or oxalates to clean and polish the monument surface (Alessandrini et al. 1989; Fassina 1989; Fassina et al. 1994). Saiz-Jimenez (1989) posited a possible origin from air pollution by assuming that oxalic acid

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Address correspondence to Dr. Maria Pia Di Bonaventura, Research Doctor in Environmental Sciences, Department of Basic and Applied Biology, Microbiology Laboratory, University of L'Aquila, Coppito, 67010 L'Aquila, Italy.

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present in aerosols can react on the surfaces of marble and limestone monuments to yield oxalates.

Von Liebig was the first to suggest its natural origin, by lichen exudation (quoted in Fassina 1989). Several authors have since maintained this hypothesis, attributing oxalate production to the various microorganisms inhabiting the monument surface, such as lichens, fungi, algae, and bacteria (Del Monte et al. 1987; Del Monte and Ferrari 1989; Chiari et al. 1989; Johnston and Vestal 1993). Many of such microorganisms produce oxalic acid in vitro and in vivo. However, the role of oxalate-producing microorganisms is still controversial because they can also play a role in the deterioration of monument surfaces (Krumbein 1988; Krumbein et al. 1989; Anagnostidis et al. 1991; Salvadori et al. 1994).

Microbial communities inhabiting monument surfaces are composed of different microorganisms, such as phototrophic, chemolithotrophic, and chemoorganotrophic bacteria (Bech-Andersen and Christensen 1983; Warscheid et al. 1988; Urzì et al. 1989), and it is almost impossible to separate individual actions from the integrated actions of the whole community (Palmer and Hirsch 1991; Urzì et al. 1992a). Oxalate production in vitro by microorganisms does not demonstrate that this acid is also produced in vivo, and it is necessary to consider many different aspects besides the physiological one, such as changes in climatic conditions during preceding centuries, essentially unknowable surface treatments in the past, and more recently air pollution (Urzì et al. 1992b).

We present here the results of research conducted on production of oxalic acid and other organic acids by bacterial communities developed in vitro over 7 months of incubation in different physical conditions (in light and in dark). These cultures originated from samples collected from stone surfaces of two historical buildings, located in L'Aquila and Camerino (province of Macerata), Italy. Oxalate accumulation by both communities was evaluated in a context of global metabolic activities that could eventually lead to either the degradation or the protection of the surface itself (Di Bonaventura 1995).

Materials and Methods

Cultures and Media

Samples from stone surfaces were aseptically collected from two historical buildings, one in L'Aquila, from a limestone surface of the "Palazzetto dei Nobili" facade before a recent restoration, and the other in Camerino, from a sandstone surface of the Cathedral portal. The samples were inoculated into an enriched medium (EM) having the following composition (g/L): $(NH_4)_2SO_4$, 0.22; KH_2PO_4, 1.2; $MgSO_4$, 0.23; yeast extract, 0.05; glucose, 67.0; and $CaCO_3$, 50.0. After sterilization of the medium, when it had cooled down, 3.3 ml of sterile solution of $FeCl_3$ (0.1 M) was added. For solid media, 2% agar (Merck) was added. Liquid cultures were kept statically in Erlenmeyer flasks at room temperature either in the dark or in the light. Samples for HPLC analyses were collected after 0.5, 1, 3, 4, 5, 6, and 7 months.

Total Nitrogen

Bacterial cultures were centrifuged at 7000*g* for 10 min, pellets were dried at 85°C for 24 h, and total N was analyzed with an automatic N-analyzer (LECO FP-228) as follows: Aliquots of 0.2 g (for each sample) were heated at 950°C by a resistance furnace in a stream of purified O_2. The gas produced by the combustion was carried by He to a thermal conductivity cell for N_2 measurement (Gallaher et al. 1976; Bremner 1996).

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HPLC Analyses

Identification and quantification of organic acids excreted in the culture medium were performed as follows: Samples were treated with 37% HCl to pH 1.8 (to solubilize residual $CaCO_3$) and filtered through a 0.22-μm (pore-size) membrane to remove both microorganisms and suspended particles. Organic acids were analyzed on a Bio-Rad Aminex HPX-87H cation-exchange column (300 × 7.8 mm) in the hydrogen form at 65°C. The eluent was 5% CH_3CN in 5 mM H_2SO_4 at a flow rate of 0.6 ml/min. Sample size was 20 μl, and detection was by measuring UV absorbance at 210 nm. Glucose was analyzed on a Bio-Rad Aminex HPX-87C cation-exchange column (300 × 7.8 mm) in the calcium form at 85°C. The eluent was H_2O at a flow rate of 0.6 ml/min. Sample size was 20 μl, and detection was by measuring the refractive index (Del Gallo and Haegi 1990; Dutton et al. 1991; Krausse and Ullmann 1991).

Isolation and Identification of the Microorganisms

The microorganisms present in the different cultures were isolated on both Nutrient Agar and Todd Hewitt medium for the isolation of aerobic and anaerobic heterotrophic bacteria, respectively. The modified Jensen medium was utilized to isolate actinomycetes. The analyses were carried out following the standard methods of the Italian Commissione NORMAL (1990). Characterization and classification of the isolated bacterial strains were carried out according to cultural, physiological, enzymatic, and biochemical analyses reported in *Bergey's Manual of Determinative Bacteriology* (Buchanan and Gibbons 1989).

Test for Carbonate Solubilization

Carbonate solubilization capacity of both single isolates and mixed cultures was analyzed on EM that had been supplemented with 2% agar and 2.5% $CaCO_3$ at 30°C for 7 days. Excretion of organic acids by the cultures, monitored by a decrease in pH, led to a quantifiable carbonate solubilization by forming a clear halo around the colony (Martino et al. 1992).

Results

HPLC analyses revealed that all mixed cultures produced oxalic acid in different amounts. The mixed culture isolated from the "Palazzetto dei Nobili" and incubated in the light released increasing amounts of this organic acid in the flask. The highest production appeared at the fourth month of incubation (Figure 1).

This L'Aquila culture also excreted other organic acids such as citric, pyruvic, succinic, acetic, and, to a small extent, propionic acid. Lactic acid was also excreted in very high, but fluctuating, amounts (Table 1). The linear correlation of the amounts of the different acids excreted in the medium revealed a good correlation between oxalic acid production and production of citric, pyruvic, succinic, acetic, propionic, and lactic acids (Table 2). During the first 3 months of incubation, glucose consumption was low: 54.0 g/L was observed in the medium after the first month and 43.0 g/L after the third. All the glucose (results not shown) was consumed during the following month.

The cultures isolated from the Cathedral of Camerino were incubated in the light and in the dark. Oxalic acid production became significant—similar to the culture isolated in L'Aquila—after the third month of incubation, showing two peaks at the fourth and fifth months (Figure 2). Although no substantial differences were observed between the production trends for cultures from this source in the dark or in the light, the total amount produced in the dark was higher than that produced in the light after 7 months of incubation.

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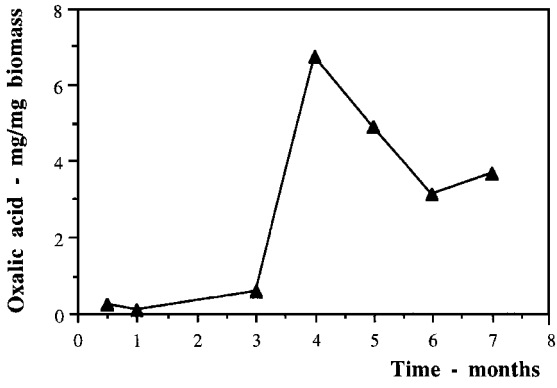

FIGURE 1 Oxalic acid production during 0.5–7 months of incubation of mixed bacterial cultures isolated from the "Palazzetto dei Nobili" (L'Aquila, Italy).

TABLE 1 Organic acid production by mixed bacterial populations isolated on stone surfaces of the "Palazzetto dei Nobili" (L'Aquila, Italy)

Months	OX	CIT	PYR	SUC	LAC	ACE	PRO
0.5	0.24	0.33	0.13	0.89	23.25	1.05	0.76
1	0.09	1.08	2.94	2.04	19.61	4.77	0.24
3	0.57	5.89	4.78	2.74	18.05	7.22	0.37
4	6.73	16.48	11.31	6.02	97.60	13.84	1.19
5	4.87	15.16	5.83	6.44	58.47	8.70	0.73
6	3.10	8.76	3.78	2.78	11.51	5.41	0.42
7	3.65	12.11	3.07	5.27	14.95	6.64	1.15

Data are expressed as mg/mg of biomass during a period of 0.5–7 months and are the average of three different analyses.

TABLE 2 Coefficients of linear regression between oxalic acid and organic acids produced by mixed bacterial populations isolated from the "Palazzetto dei Nobili" in L'Aquila and the Cathedral of Camerino

Other organic acid	Linear correlation with oxalic acid		
	"P. dei Nobilis"	Cathedral of Camerino	
	light	Light	Dark
Citric	0.92	0.08	0.71
Pyruvic	0.65	0.14	0.56
Succinic	0.81	0.11	0.37
Lactic	0.60	0.23	0.05
Acetic	0.70	0.01	0.60
Propionic	0.50	0.82	—

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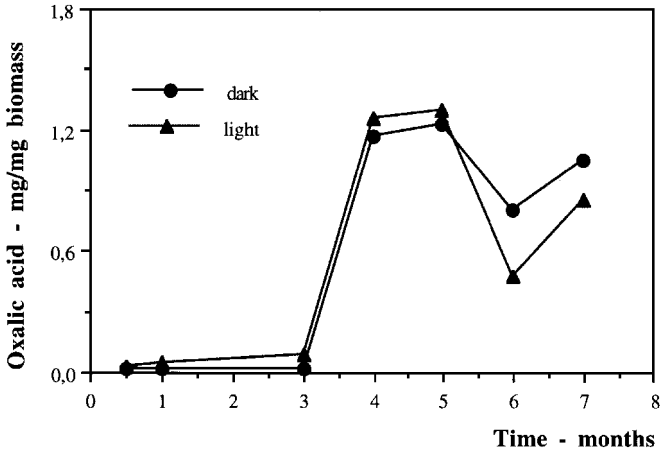

FIGURE 2 Oxalic acid production during 0.5–7 months of incubation in the light or in the dark of mixed bacterial cultures isolated from the Cathedral of Camerino (Macerata, Italy).

Differences in patterns of organic acids excretion were observed between the two conditions. In particular, the cultures incubated in the dark showed the highest production of lactic acid and no excretion of propionate (Table 3). Cultures incubated in the light showed a trend similar to the cultures isolated in L'Aquila.

The linear correlation of the amounts of the different acids excreted in the medium revealed a good correlation between the production of oxalic acid and that of citric, pyruvic, and acetic acids when the Camerino cultures were incubated in the dark. When incubated

TABLE 3 Organic acid production by mixed bacterial populations isolated on stone surfaces of the Cathedral of Camerino (Macerata, Italy) and incubated in the light or in the dark

Months	OX	CIT	PYR	SUC	LAC	ACE	PRO
Dark							
0.5	0.02	0.04	0.12	0.31	5.70	0.58	0
1	0.02	0.74	0.14	0.26	10.46	0.78	0
3	0.02	0.81	0.30	1.07	10.82	2.05	0
4	1.17	4.31	0.45	1.62	21.21	3.37	0
5	1.23	3.35	2.18	1.50	8.40	3.69	0
6	0.80	1.62	1.88	3.64	92.19	5.67	0
7	1.05	1.45	2.07	5.03	12.18	5.90	0
Light							
0.5	0.03	1.14	0.36	1.04	1.41	5.32	0.53
1	0.05	1.41	0.75	1.50	5.52	5.92	0.85
3	0.09	1.33	0.19	0.36	8.99	20.72	4.13
4	1.26	4.90	2.44	2.68	16.56	15.05	7.90
5	1.29	0.12	0.09	0.33	7.76	9.65	6.33
6	0.47	0.13	0.07	1.34	4.38	9.94	4.40
7	0.85	0.52	0.18	2.88	12.60	2.39	7.25

Data are expressed as mg/mg of biomass during a period of 0.5–7 months and are the average of three different analyses.

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TABLE 4 Solubilization activity of various strains isolated from the "Palazzetto dei Nobili" in L'Aquila and from the Cathedral of Camerino

	"Palazzetto dei Nobili"		Cathedral of Camerino	
Strains	Genus	Solubilization	Genus	Solubilization
1	*Bacillus*	+	*Alcaligenes*	+++
2	*Flavobacterium*	s.p.	*Alcaligenes*	+
3	*Flavobacterium*	+++	*Actinomyces*	s.p.
4	*Flavobacterium*	+++	*Bacillus*	+
5	*Flavobacterium*	+++	*Micrococcus*	s.p.
6	*Nocardia*	−	*Micrococcus*	s.p.
7	*Nocardia*	−	*Flavobacterium*	++
8	*Nocardia*	+	*Flavobacterium*	++
9	Not identified	s.p.	Not identified	+
10	Not identified	+	Not identified	++
11	Not identified	+	Not identified	s.p.

Symbols: −, negative; +, positive for solubilization; ++, high solubilization; +++, very high solubilization; s.p., spotted solubilization.

in daylight, the better correlation was of oxalic acid with propionic acid (Table 2). Glucose utilization showed different trends in the light and in the dark. In one case, the Camerino cultures utilized almost two-thirds of the glucose after the first month; glucose disappeared completely in the medium after the third month. In the other case, glucose measured in the medium was 41.0 and 36.0 g/L after the first and the third months of incubation, respectively, and then remained unchanged up to the end of the incubation.

Several bacteria were isolated from both cultures, among them species identified as *Alcaligenes* sp., *Actinomyces* sp., *Bacillus* sp., *Flavobacterium* sp., *Micrococcus* sp., and *Nocardia* sp. Both rock materials showed similarity in the composition of microbial populations. Almost all isolates showed carbonate-solubilization capability (Table 4), *Flavobacterium* sp. and *Alcaligenes* sp. being particularly effective (Figure 3). *Nocardia* was the only strain that showed very poor or no solubilization activity. Mixed cultures solubilized calcium carbonate to greater extents than the pure cultures did (Figure 4).

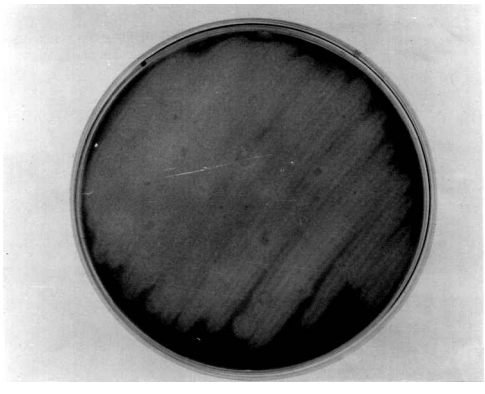

FIGURE 3 Calcium carbonate solubilization by *Alcaligenes* sp. isolated from the Cathedral of Camerino.

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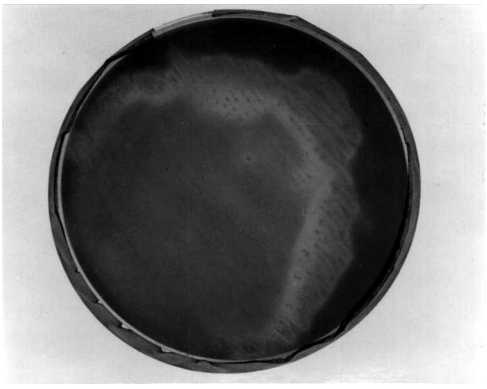

FIGURE 4 Calcium carbonate solubilization by mixed bacterial cultures isolated from the "Palazzetto dei Nobili."

Discussion

These results demonstrate that different amounts of oxalic acid are produced and released by the examined microbial populations. This acid is produced in higher amounts in bacterial cultures isolated from the "Palazzetto dei Nobili," in comparison with the other cultures. Besides oxalate, several other organic acids were excreted into the medium in considerable quantities, among them citric, pyruvic, succinic, lactic, acetic, and propionic acids. The amounts of the different acids varied with the duration of incubation in the sample collected from the "Palazzetto dei Nobili" and with the different conditions of incubation (light or dark) in the cultures from the Cathedral of Camerino. Oxalic acid, however, was released in comparatively lesser amounts than the other organic acids and was significantly accumulated only after the third month of incubation. Among the organic acids, lactic acid was excreted at the highest amounts, in particular in the cultures isolated from the "Palazzetto dei Nobili" and in those from Camerino incubated in the dark.

All data were statistically examined, and the factors considered were analyzed for their influence on the presence of oxalic acid. Some correlations that can clarify the production of this acid are as follows:

- In the case of the "Palazzetto dei Nobili," the positive correlation between oxalic acid and the citric, pyruvic, succinic, and acetic acids released into the medium seems to indicate an aerobic metabolism of the cultures, possibly connecting the excretion of this specific acid to the Krebs cycle. However, because oxalic acid is also correlated to lactic and propionic acids, we can also hypothesize a fermentative metabolic contribution.
- The cultures isolated in Camerino, when incubated in the light, give a significant correlation of oxalic acid only with propionic acid, suggesting that Krebs cycle intermediates are not involved.
- There is a good correlation between oxalic acid accumulation and the presence of pyruvic, citric, and acetic acids when these cultures are incubated in the dark. Succinic and lactic acids seem not to be related to oxalate. Propionic acid is not excreted at all.

With regard to the consumption of glucose in the medium, the bacterial cultures showed different behaviors. The microbial population sampled from the "Palazzetto dei Nobili" utilized the carbon source very slowly during the first 3 months. Glucose disappeared after the fourth month, and the biomass (results not shown) reached its maximum amount at the

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end of the incubation. In contrast, the cultures collected from the Cathedral of Camerino (and grown in the light) utilized the glucose rapidly—no glucose was found at the third month—and they reached a biomass higher than that of the cultures incubated in the dark. Glucose utilization, and thus biomass accumulation, were lower in the cultures incubated in the dark. However, the oxalic acid production was similar under both incubation conditions (light or dark).

The release of organic acids in a medium containing organic matter and calcite powder, after incubation with the bacterial populations isolated from stone surfaces, gives indirect evidence of the events likely to originate the accumulation of calcium oxalates on a monument surface. The excessive amounts of calcium carbonate play a determinant role through the formation of calcium salts of these acids, each one characterized by different dissociation constants and solubilities in water (Merck Index 1989). Calcium oxalates, including the ones found in oxalate films (whewellite and weddellite), are characterized by a very low ionization constant and by being almost insoluble in water. These properties are not present to the same extent in the calcium salts of other organic acids. Consequently, a differential leaching effect can be postulated that leads to an increasing concentration of oxalates while the salts of other organic acids are progressively removed.

The correlations between various amounts of organic acids with oxalate ions were calculated and some of them were statistically significant. This indicates a possible mechanism by which the presence of various organic acids is part of the bacterial metabolic pathway and belongs to a general pattern in which the acids are released and reutilized according to the needs of the cells. When conditions favoring the release and accumulation of oxalates are present, the pool of organic acids related either to aerobic or to anaerobic metabolism is a candidate for establishing an organic reserve for oxalate accumulation.

As to the systematics of the isolated bacteria, we note the ubiquitous presence of *Bacillus* and *Flavobacterium*, which are already known as colonizers of the monument surface. *Flavobacterium* produces pigments that give a yellowish-orange color to the area around the colonies. Other strains able to contribute to such a color alteration belong to *Micrococcus* sp., which was found in the cultures isolated from Camerino. As to the capability of solubilizing $CaCO_3$, we note that this property is characteristic both of single strains—*Flavobacterium* sp. and *Alcaligenes* sp., in particular—and of mixed cultures. A contribution to such activity could derive from specific actions of the bacteria or from the release of metabolic byproducts ranging from carbon dioxide to the above-reported organic acids.

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