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# Is the contribution of bacteria to terrestrial carbon budget greatly underestimated?

**Abstract** Some commonly found species of soil bacteria use low molecular weight organic acids as their sole source of carbon and energy. This study shows that acids such as citrate and oxalate (produced in large amounts by fungi and plants) can rapidly be consumed by these bacteria. Two strains, *Ralstonia eutropha* and *Xanthobacter autotrophicus*, were cultured on acetate- and citrate-rich media. The resulting $CO_2$ and/or $HCO_3^-$ reacted with calcium ions to precipitate two polymorphs of calcium carbonate ($CaCO_3$), calcite and vaterite, depending on the quantity of slime produced by the strains. This production of primary calcium carbonate crystals by oxalate- and citrate-degrading bacteria from soil organic carbon sources highlights the existence of an important and underestimated potential carbon sink.

## Introduction

In soils, low molecular weight organic acids are usually associated with microbial decay of organic matter and/or root secretions in the rhizosphere (see Jones 1998 for a review). Acetic, citric and oxalic acids produced by fungi during organic matter degradation are able to interact with metal ions and particularly with calcium (Ca) and iron (Fe). As fungi constitute an important and ubiquitous biomass in soils and sediments (Gobat et al. 1998; Verrecchia 2000), these three acids are believed to play an important role in various biogeochemical cycles (Cochrane 1958; Verrecchia 1990; Gadd 1999), e.g. the dissolution of phosphate minerals such as variscite ($AlPO_4 \times 2H_2O$) (Illmer et al. 1995).

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Low molecular weight organic acids, such as acetate, glyoxylate, succinate, malate and citrate, are used as the sole carbon and energy sources by many bacteria and are metabolized through the tricarboxylic acid and glyoxylate cycles (Prescott et al. 1995; Lengeler et al. 1999). Oxalate is used as the sole carbon and energy source by a limited number of bacterial species. It is assimilated through either the serine pathway or the glycolate pathway (Tamer and Aragno 1980). Due to its high oxidation degree and low molecular weight, the growth yield is low, about 2.5 g/mol. Despite the low solubility of metal oxalates ($Kps=4\times10^{-9}$ for Ca oxalate), they can be dissolved by oxalate-utilizing bacteria (Chandra and Shethna 1975; Dijkhuizen et al. 1977; Friedrich et al. 1979; Tamer and Aragno 1980). Generally, the metabolic role of oxalate in other bacteria remains unclear, but it is assumed to be used in pH regulation, energy production from glyoxylate and in aluminum detoxification by some bacteria (Hamel et al. 1999; Tanner and Bornemann 2000). Another reason for bacterial degradation of oxalate is its detoxification, as it can easily become toxic for many strains. Detoxification may occur, as it does in some plants using an oxalate oxidase, which produces $CO_2$ and $H_2O_2$ from oxalate (Koyama 1969).

Bacterial heterotrophic aerobic catabolism results in general in the complete oxidation of the organic electron and energy source, resulting in the production of $CO_2$ and/or $HCO_3^-$ ions. This is the case with citrate and oxalate (Blackmore and Quayle 1970; Prescott et al. 1995; Lengeler et al. 1999). In natural environments, carbonate ions will rapidly react with $Ca^{2+}$ to precipitate $CaCO_3$ when the required conditions are reached.

The aim of this study is to show that low molecular weight organic acids such as citrate and oxalate produced in large amounts by fungi and plants can rapidly be consumed by soil bacteria. This consumption results in $CO_2$ and/or $HCO_3^-$ production and, after reacting with calcium ions, Ca carbonate is precipitated. Particular attention is paid to Ca oxalate crystals (weddellite and whewellite) commonly associated with fungi (Clémençon 1995; Dutton and Evans 1996) and with

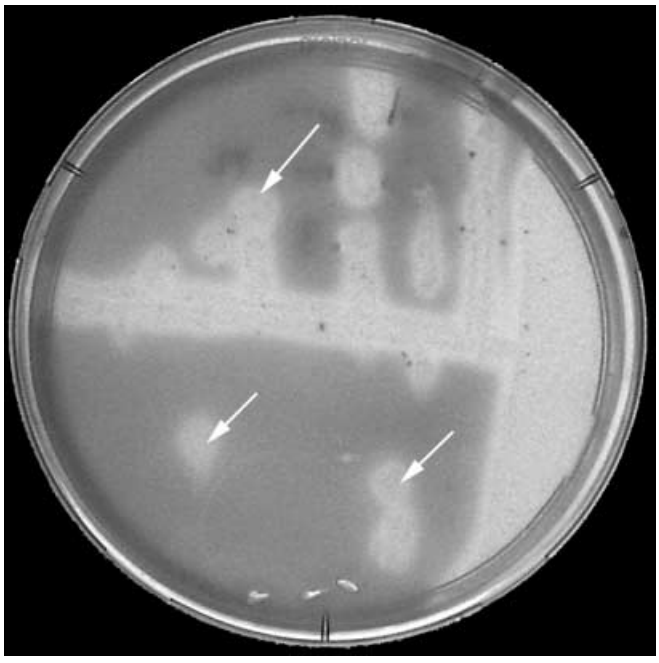

**Fig. 1** Clear zones (*arrows*) obtained during oxalate dissolution by *Ralstonia eutropha* colonies

some plants (Horner and Wagner 1995; Franceschi 2001). These crystals may constitute a large metastable substrate for bacteria.

## Materials and methods

The bacteria used in this study were *Ralstonia eutropha* H16 (syn: *Alcaligenes eutrophus*, DSM: 428, ATCC 17699) and *Xanthobacter autotrophicus* (DSM 432, ATCC 35674). These bacteria are ubiquitous and most easily found in oxalate-rich litters such as those associated with *Oxalis*, *Rumex*, *Rheum* and *Eucalyptus*. These two bacterial strains were obtained from the Neuchâtel University Microbiology Laboratory (LAMUN). Both species metabolize oxalate through the glycolate pathway.

Bacteria were grown at 26°C on citrate containing media (Merck yeast extract 4.0 g/l; Fluka tricalcium dicitrate 2.5 g/l; Merck agar-agar 15.0 g/l) and on a Schlegel mineral medium (Aragno and Schlegel 1991) containing calcium oxalate (first layer: $Na_2HPO_4{\times}12H_2O$ 9.0 g/l, $KH_2PO_4$ 1.5 g/l, $NH_4Cl$ 1.0 g/l, $MgSO_4{\times}7H_2O$ 0.2 g/l, ammoniacal ferric citrate 0.005 g/l, $CaCl_2$ 0.01 g/l, $ZnSO_4{\times}7\ H_2O$ 50 µg/l, $MnCl_2{\times}4H2O$ 15 µg/l, $H_3BO_3$ 150 µg/l, $CoCl_2{\times}6\ H_2O$ 100 µg/l, $CuCl_2{\times}2H_2O$ 50 µg/l, $NiCl_2{\times}6H_2O$ 10 µg/l, $NaMoO_4{\times}2H_2O$ 15 µg/l, agar 15 g/l; second layer: Schlegel mineral medium to which was added calcium oxalate monohydrate 4 g/l). On both media, consumption of citrate or oxalate was indicated by the formation of a clear zone around the colonies, due to the dissolution of Ca citrate and Ca oxalate (Fig. 1). In order to obtain more $CaCO_3$ crystals, bacteria were grown on a B4 medium (Merck yeast extract 4.0 g/l; Merck calcium acetate 2.5 g/l; Merck agar-agar 15 g/l). After 20 days, crystals were collected from the bacterial colony and washed in a saturated calcium hypochloride solution (to remove organic matter) until the solution remained clear. Crystals were analyzed by X-ray diffraction (XRD) using a Scintag diffractometer, and observed with a Philips XL 30 ESEM and XL 20 SEM. The XL 30 ESEM was coupled to an EDS microprobe.

In order to monitor the conditions for crystal growth on oxalate and its effect on the medium, liquid cultures were made in a Schlegel medium (Schlegel mineral medium to which was added K oxalate 4.0 g/l) at 30°C in low agitation. Variations in the medium pH were measured with a Methrom electrode (Herisau, Switzerland). Oxalate consumption has been measured titrimetrically with $KMnO_4$ 0.02 M in the presence of $H_2SO_4$ 0.1 M. Finally, bacterial growth was measured turbidimetrically at 600 nm (using a Perkin-Elmer spectrophotometer). After total consumption of oxalate, $CaCl_2$ 1 M was added to the culture media in order to precipitate the carbonate formed. The resulting precipitate was analyzed by XRD.

## Results

$CaCO_3$ crystals were precipitated on acetate- and citrate-containing media inoculated with either *R. eutropha* or *X. autotrophicus* (Fig. 2A, B). They formed either inside or close to the colony. Large amounts of other carbonate crystals identified as calcium phosphate carbonate and magnesium phosphate carbonate by microprobe analysis have been found on the Schlegel mineral medium enriched with calcium oxalate (Fig. 2C, D).

*Ralstonia eutropha* produced colorless twin crystals ranging from 100 µm to 600 µm in size, whereas *X. autotrophicus* produced smaller brown spherulites having a maximum size of 200 µm. XRD analysis of crystals revealed that two polymorphs of $CaCO_3$ were precipitated during the growth on acetate- and citrate-rich media. *Ralstonia eutropha* preferentially precipitates calcite crystals, whereas *X. autotrophicus* mainly precipitates vaterite spherulites (Fig. 2A, B).

In addition, XRD analyses showed no difference between crystals produced on acetate and citrate media for both bacterial strains. On the Schlegel medium, similar calcium and magnesium phosphate carbonate crystals were produced by the two strains (Fig. 2C, D). XRD analyses suggest that calcium phosphate carbonate crystals may have a structure similar to rapidcreekite ($Ca_2(SO_4)(CO_3){\times}4H_2O$) (Roberts et al. 1986).

The growth of *R. eutropha* and *X. autotrophicus* in the liquid Schlegel medium containing potassium oxalate showed a rapid consumption of oxalate associated with a continuous increase in pH (Fig. 3). The pH rapidly increases during the exponential growth of the colony and continues to increase at a lower rate after the stationary phase has been reached. This can be explained by the release of metabolites during lysis of dead cells, which are probably quickly reused by living cells, after the oxalate source has been totally exhausted. Final pH after 7 days of incubation was >9.5 in each case. Calculations show that the theoretical pH should reach a value of 9.55±0.05 after total oxalate consumption, emphasizing the role of bacteria in providing the required conditions for calcium carbonate precipitation.

XRD analysis of precipitates obtained by addition of calcium chloride 1 M indicated the presence of carbonate in the media. As the Schlegel medium contains large amounts of phosphates, carbonate ions were not directly precipitated as calcium carbonate but as calcium phosphate carbonate ($Ca_{10}(PO_4)_6CO_3$). Another byproduct of the precipitation triggered by $CaCl_2$ 1 M is chloroapatite

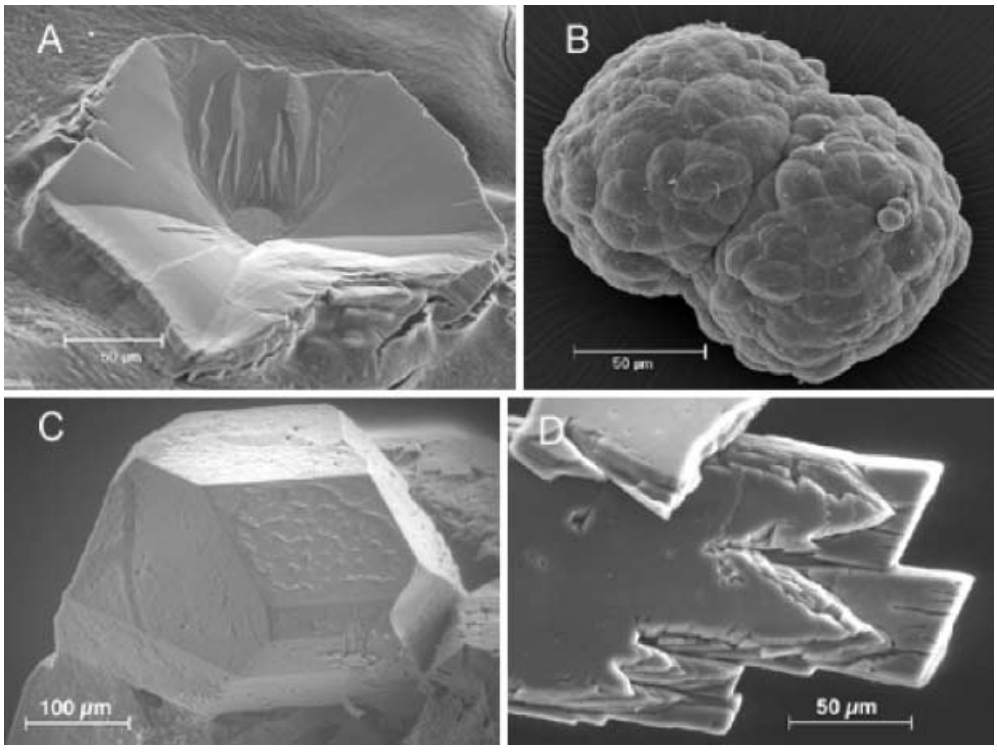


**Fig. 2** Scanning electron microscope (*SEM*) images of calcium carbonate crystals obtained in bacterial culture (**A**, **B**) and SEM view of magnesium calcium carbonate phosphate (**C**, **D**) obtained in bacterial culture (*Ralstonia eutropha* and *Xanthobacter autotrophicus*). **A** calcite crystal associated with *R. eutropha*, **B** vaterite spherulite associated with *X. autotrophicus*, **C** magnesium phosphate carbonate, **D** calcium phosphate carbonate

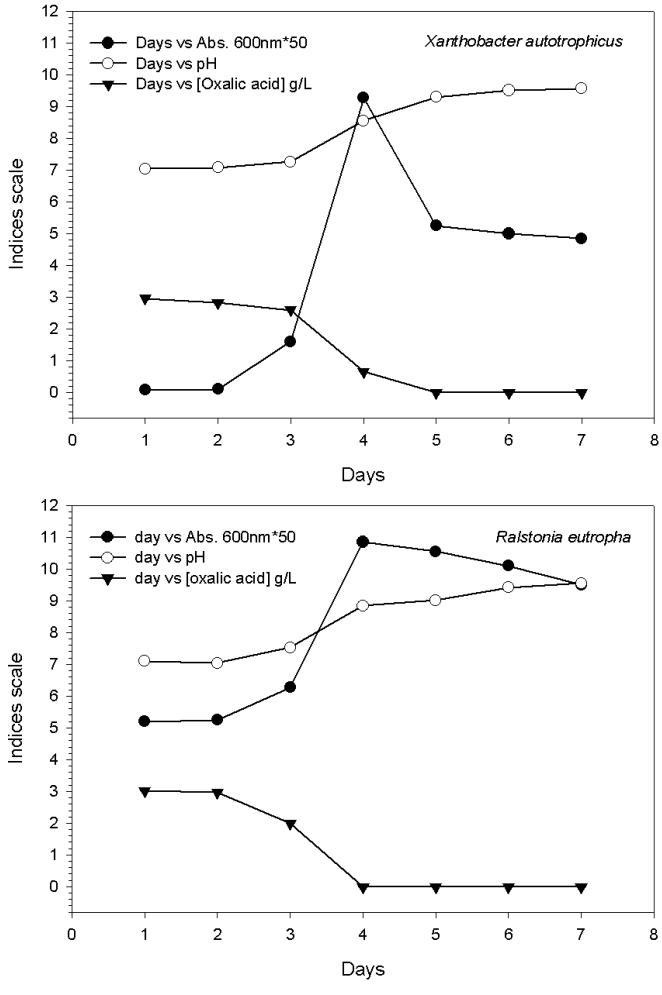


**Fig. 3** Variations in indices [cell density measured turbidimetrically at 600 nm, pH and oxalic acid concentration (g/l)] during the culture of *X. autotrophicus* (*top*) and *R. eutropha* (*bottom*) in a liquid medium

($Ca_5(PO_4)3Cl$), which is not surprising regarding the composition of the Schlegel medium. Only chloroapatite and calcium oxalate have been detected in the control solutions, highlighting the role played by bacteria in oxalate oxidation.

## Discussion

Calcium carbonate production by bacteria from low molecular weight organic acids seems to be a common and rapid process. On acetate- and citrate-containing media, results clearly show the influence of bacteria on the mineralogical nature and shape of $CaCO_3$ crystals produced on similar media. Precipitation of biogenic calcite crystals is quite common (Boquet et al. 1973; Simkiss and Wilbur 1989). However, biogenic production of vaterite has only been reported for a few groups (Lowenstam and Weiner 1989). It is known that organic matter and medium viscosity can influence $CaCO_3$ crystallization (Kitano and Hood 1965; Cailleau et al. 1979; Addadi et al. 1990; Buczynski and Chafetz 1991). This point is particularly important in this study because one of the main cultural characteristics differentiating the two strains studied is the amount of slime produced (mainly exopolysaccharides composed of glucuronic acid, glucose and mannose). Indeed, *X. autotrophicus* secretes

abundant slime, noticeably increasing the viscosity of the media (Wiegel 1991).

The influence of organic matter, exopolysaccharides and polyaspartate in the precipitation of vaterite can also be found in nature (Falini et al. 1998; Gower and Tirrell 1998). Vaterite crystals similar to those obtained in this study have recently been found in Lake Issyk-Kul (Kyrgyzstan) and have been attributed to *Synechococcus* activity in mucilagenous mats containing polyaspartate (Giralt et al. 2001). However, as there is a great diversity in the composition, chemical and physical properties of bacterial slimes (see Sutherland 2001a, b, c for a review), it is premature to conclude that every bacterial strain producing slime will precipitate vaterite crystals.

Culture in liquid and solid Schlegel oxalate media demonstrate that bacteria change the medium conditions, allowing calcite precipitation. Rapid consumption of oxalate in liquid media and production of calcium and magnesium phosphate carbonate on solid media suggests that large amounts of calcium oxalate or free oxalic acid are probably rapidly transformed into secondary Ca carbonate in soils. This hypothesis is supported by biogeochemical studies (Verrecchia and Dumont 1996), soil biology (Cromack et al. 1977) and soil micromorphology (Verrecchia 1990; Monger et al. 1991).

## Conclusion

$CaCO_3$ crystals can easily be produced by two common oxalate-degrading bacteria, *R. eutropha* and *X. autotrophicus*. Crystals, and particularly vaterite spherulites, can be identified in soils where soil bacteria have been involved in $CaCO_3$ precipitation. The two bacteria described in this study use oxalate and citrate as a carbon and energy source. The biogeochemical implications of oxalate consumption and carbonate production by bacteria in soils and surficial sediments emphasizes the existence of an important and underestimated potential carbon sink.

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