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Surface Enhancement of Sporulation and Manganese Oxidation by a Marine Bacillus

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In a seawater medium containing 0.005% yeast extract and 0.005% peptone, a marine bacillus, SG-1, sporulated only when associated with solid surfaces. The spores (rather than the vegetative cells) were responsible for the oxidation of manganese, and the degree of sporulation was determined by the surface area available rather than by the chemical nature of the clay or silica surface used.

Although it has been known for some time that many genera of bacteria can catalyze manganese oxidation (7) and that solid MnO_2 can cause some marine bacteria to oxidize adsorbed manganese (1, 2), only recently has the effect of other solid surfaces been investigated. Nealson and Ford (8) have demonstrated that the oxidation of Mn^{2+} by a marine bacillus, SG-1, is enhanced when surfaces other than MnO_2 are used. In addition, Rosson and Nealson (9) have shown that SG-1 has the ability to sporulate and that the spores (rather than vegetative cells) oxidize manganese. As a result SG-1, like many other bacteria (6), may undergo extensive physiological changes when associated with surfaces. We report here how suspensions of silica and laboratory clays control the sporulation of SG-1 and discuss the possible significance that this process may have in the promotion of manganese oxidation in nature.

MATERIALS AND METHODS

Surface area of silica and clays. The Brunauer-Emmet-Teller method was used to determine surface areas. The method, which is discussed in detail by Gregg and Sing (3), is based on the fact that dry surfaces will adsorb liquid nitrogen and be completely covered, provided that the amount of nitrogen present is a specified small fraction of the saturated vapor pressure of liquid nitrogen.

Three laboratory clays and a purified amorphous silica were analyzed with a Chromosorb surface area analyzer, and their surface areas are expressed as surface area per gram of sample. The clays and silica were chosen to cover as wide a range of surface areas and surface chemistries as possible. The clays were kaolinite (University of Missouri no. KGA2), illite (Ward's no. 36), and montmorillonite (Ward's no. 24), and the silica was prepared by drying an amorphous silica suspension.

Growth procedures. Cells were grown to an optical density of 0.4 at 550 nm, and 0.1 ml of this inoculum was introduced to 100 ml of sterile M medium (0.05 g of Difco yeast extract and 0.05 g of Difco peptone per liter of natural seawater) containing previously sterilized clay or silica. In addition, 1.35 μCi (3.1 pmol/100 ml) of carrier-free $^{54}MnCl_2$ (New England Nuclear Corp.) was added as a tracer to follow the binding and oxidation of manganese. The concentration of unlabeled manganese in the seawater was between 0.2 and 0.6 nmol/100 ml (5).

Manganese binding was followed at room temperature (24 ± 2°C) with a range of clay or silica concentrations (Table 1). The amount of clay or silica added to each culture was determined by our desire that a wide range of surface areas be exposed to SG-1 and by the necessity that the suspensions remain well mixed while the cultures were shaken. For each culture grown with surfaces, a poison control was included in which 1 mg of sodium azide was added per ml of culture; additional, uninhibited cultures were also grown without surfaces.

Samples of 1 ml were removed aseptically each day and washed through 0.2-μm membrane filters (Gelman) with 2 ml of sterile seawater to remove manganese associated with the filters. The filters and filtrates from each of the samples were then assayed for their ^{54}Mn content with a Beckman Biogamma II counter. The filters were also washed with 2 ml of 10 mM $CuSO_4$ (pH 4.8) to determine the manganese adsorbed onto cells and with 1 ml of 0.02% hydroxylamine-hydrochloride followed by 2 ml of 10 mM $CuSO_4$ to determine the manganese oxidized (8). Additional 3-ml samples of the sediment suspensions were sampled daily and frozen for dipicolinic acid (DPA) analysis.

DPA assay. DPA is a substance unique to bacterial spores (11) and is an excellent indicator of their presence in a culture. DPA was freed from spores by the method of Janssen et al. (4), where 3-ml samples were autoclaved for 15 min at 15 lb/in^2 to rupture the spores, and the cooled suspensions were acidified with 0.06 ml of 1 M acetic acid for 1 h to allow insoluble material to aggregate. The insoluble material was then removed by centrifugation at 1,500 × *g* for 10 min. After centrifugation, 2 ml of supernatant was drawn off each sample and assayed for DPA by the method of Scott and Ellar (10), which is based on determining the difference spectrum of calcium DPA and DPA^{2-}. By the same procedure, DPA was converted to spore number using suspensions of SG-1 spores that were prepared and cleaned by the methods of Rosson and Nealson (9). The clean spores were counted micro-

TABLE 1. Surface areas of clays and silica used in the cultures of SG-1

Sample	Specific surface area (m^2/g)	Clay or silica concn (g/100 ml)	Specific surface area in cultures (cm^2/ml)
Kaolinite (University of Missouri no. KGA 2)	18.4	0.250	460
Illite (Ward's no. 36)	39.0	0.250	975
Montmorillonite (Ward's no. 24)	106.3	0.043	457
	106.3	0.250	2,658
	106.3	0.300	3,190
Amorphous silica	41.0	0.250	1,025
	41.0	0.500	2,050
	41.0	1.000	4,100

scopically and then lysed as described above to determine DPA per spore.

RESULTS

The range of surface areas used in the cultures was relatively large (Table 1), and the values of surface area per gram were in good agreement with published data (12). Approximately six times less montmorillonite was required to produce the same surface area per milliliter as a given weight of kaolinite, and about two times less illite or silica was required to produce the same effect.

Manganese was not bound when surfaces were absent from the incubations (Fig. 1); as the surface area was increased, there was a similar increase of the eventual rate of manganese removal from solution by uninhibited cultures. Manganese binding did not vary uniformly with time; there was an initial rapid removal of manganese from solution, followed by a period of 4 to 6 days before this process resumed (Fig. 1). When the filtered cells were washed with 10 mM $CuSO_4$, it was found that greater than 95% of the manganese bound during the first day was adsorbed rather than oxidized. In contrast, when filters were washed with 0.02% hydroxylamine-hydrochloride followed by 10 mM $CuSO_4$, greater than 80% of the manganese bound from 4 to 6 days was oxidized rather than adsorbed. Manganese binding during the first day was the same in the uninhibited cultures and poison controls (Fig. 2), but there was no resumption of binding by the poison controls after 1 day. There was no apparent difference in the magnitude or pattern of manganese binding by uninhibited cultures when the weights of clay or silica were adjusted to produce the same surface area per milliliter (Fig. 3).

The resumption of manganese binding by the uninhibited cultures after 4 to 6 days was accompanied by the first appearance and an increase of the DPA recovered (Fig. 4). DPA was not recovered from uninhibited cultures without surfaces, and the maximum DPA recovered was related to

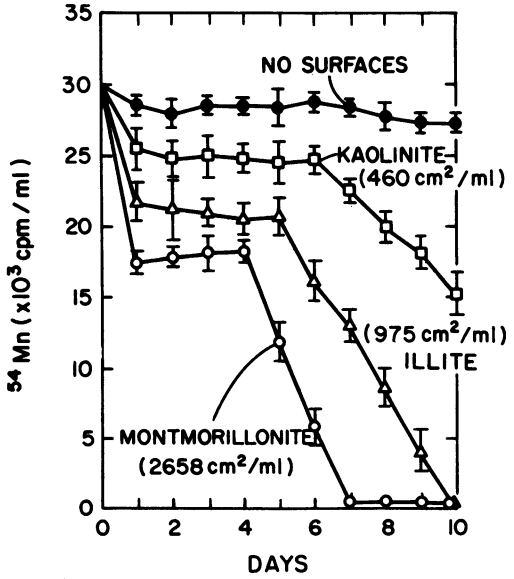

FIG. 1. Uptake of $^{54}Mn^{2+}$ by cultures of *Bacillus* sp. strain SG-1 grown with clays of different surface areas. The surface areas are expressed as surface area per milliliter, and the error bars delineate two times the standard deviation around mean uptake values.

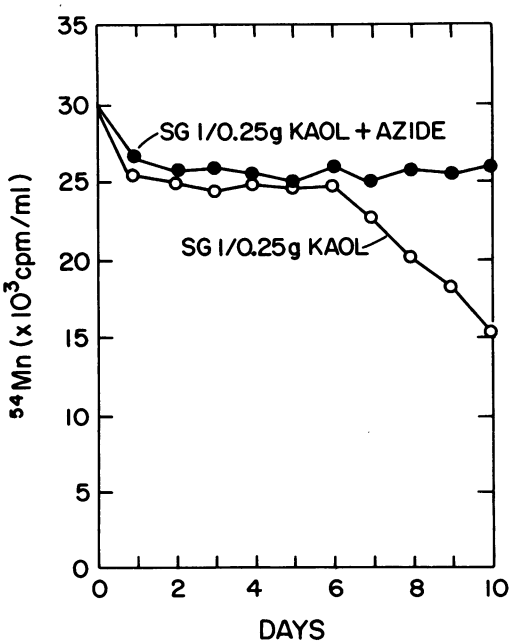

FIG. 2. Uptake of $^{54}Mn^{2+}$ by SG-1 cultures containing kaolinite in the presence and absence of azide.

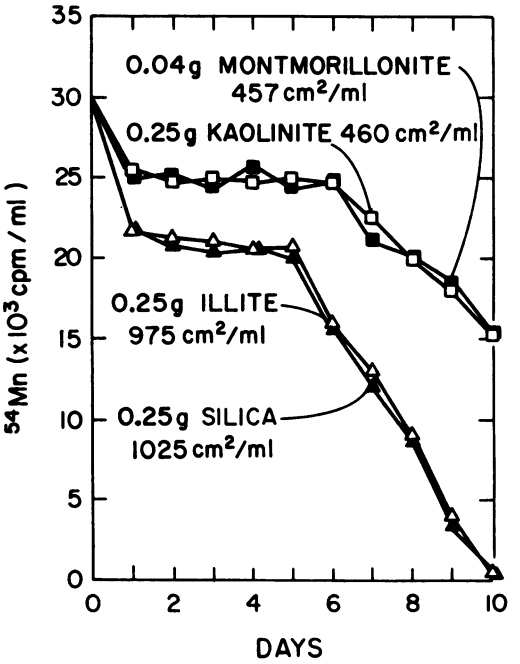

FIG. 3. Uptake of $^{54}Mn^{2+}$ by cultures of SG-1 with different weights of clay and silica. The amounts of clay or silica were adjusted to produce two sets of incubations with matched surface areas.

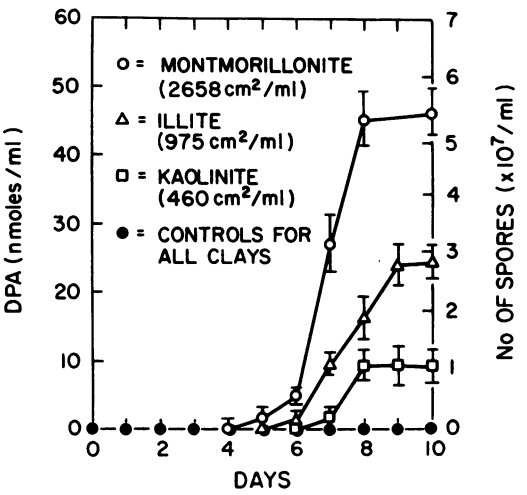

FIG. 4. DPA recovered from autoclaved culture samples. Spore number was determined on the basis of DPA recovered from a reference spore suspension that was prepared by the methods of Rosson and Nealson (9).

the surface area per milliliter added (Fig. 5). Almost identical amounts of DPA were released when the weights of clay or silica were adjusted to produce the same surface areas per milliliter (Fig. 5), but DPA recovery and manganese binding eventually levelled off, even though the surface area per milliliter increased (Fig. 6).

DISCUSSION

The initial binding of manganese by both live and inhibited cultures (Fig. 1) was the result of abiotic processes such as the adsorption of Mn^{2+} onto clay or silica surfaces, but the resumption of binding after 4 to 6 days could only have been biological in origin. The resumption of manganese binding accompanied by manganese oxidation (Fig. 1) was closely associated with the onset and maintenance of sporulation (Fig. 4). This is in agreement with the data of Rosson and Nealson (9) which show that the spores themselves (rather than the vegetative cells) are responsible for the oxidation of manganese, even at the very low concentrations of Mn^{2+} used in this work (0.2 to 0.6 nmol/100 ml).

M medium was used in a seawater rather than an artificial seawater base in an attempt to reproduce a natural system, which normally has low levels of labile organics and trace amounts of manganese in solution (5). The number of spores produced in this nutrient-deficient environment (Fig. 4) was determined by the surface area available to induce sporulation and was apparently independent of the chemical nature of the surfaces (Fig. 5). Nealson and Ford (8) have suggested that the vegetative cells of SG-1 could attach to surfaces in an attempt to secure organic nutrients. We can now add that if the cells do eventually run out of a specific nutrient, they can then sporulate on the surfaces. Neither

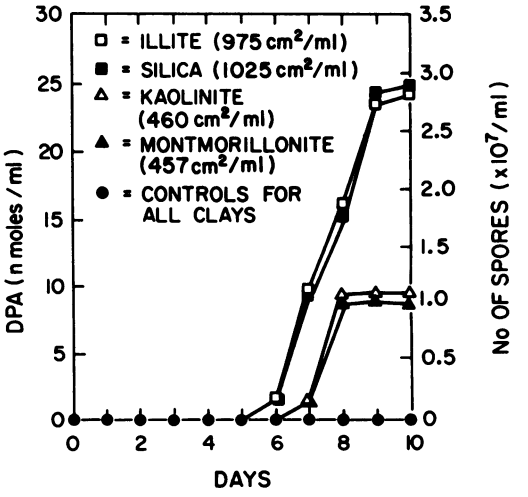

FIG. 5. Effect of clay or silica surface area on the DPA recovered from autoclaved culture samples. The amounts of clay or silica were adjusted to produce two sets of cultures with matched surface areas. Data from cultures inhibited with azide are plotted as closed circles.

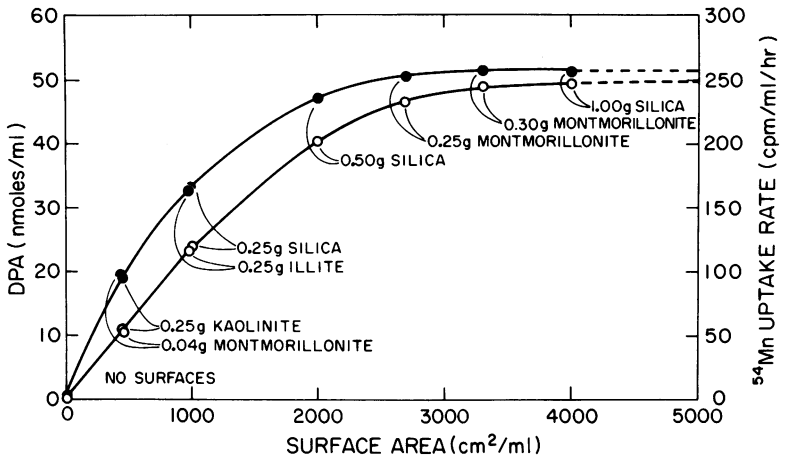

FIG. 6. Effect of surface area on DPA recovery (○) and the rate of $^{54}Mn^{2+}$ uptake (●).

of these suggestions is based on sufficiently precise data, and both remain to be proven, but it is clear from Fig. 4 and 5 that surfaces were a necessary prerequisite for sporulation in M medium. Microscopic observations have shown that SG-1 cells died and did not sporulate when surfaces were absent in low organic media, even when the cultures were amended with micromolar or millimolar manganese. This is in distinct contrast to the data of Rosson and Nealson (9), which show that with a sufficiently rich organic medium and only trace amounts of manganese, sporulation will eventually occur in the presence or absence of surfaces. The contention that an organic nutrient on surfaces may control the extent of sporulation is further supported by Fig. 6, which shows that the sporulation of SG-1 could only be brought to a certain level in M medium. After this maximum response was attained, there was no further enhancement of sporulation as surface area increased, presumably because there was a limited supply of organics for cell and spore production on surfaces.

Aquatic environments such as that found in Saanich Inlet, British Columbia, may be ideal places to look for this type of bacterial activity. Emerson and co-workers (2a) have shown that a peak of manganese-oxidizing activity is associated with a high concentration of suspended particulates at a well-defined aerobic-to-anaerobic transition in the inlet. This may be a zone where surfaces induce sporulation, and transmission electron micrographs of the particulate fraction (7) reveal spore-like structures encased in manganese precipitates. The oxidation of manganese by spores may be even more important in sediments where excess surface area is available to promote sporulation.

The overall environmental significance of spore-forming bacteria to the oxidation of manganese remains unknown. However, the fact that surfaces can induce sporulation (in, admittedly, only one marine bacillus) illustrates how sediments may provide environments for which spore formers are suited. Nealson and Ford (8) have already suggested that the methods normally used to isolate manganese oxidizers may have led to the systematic underestimation of those requiring surfaces. For the same reasons we suggest that spore formers, whose spores are primarily associated with surfaces, may play an equally underestimated role in the oxidation of manganese.

ACKNOWLEDGMENTS

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