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EXPERIMENTAL ARTICLES

Effect of Congo Red on the Motility of the Bacterium *Azospirillum brasilense*

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Abstract—In semiliquid laboratory media, the bacterium *Azospirillum brasilense* migrates with the formation of swarming rings. It is demonstrated that adsorption of the sulfonated azodye Congo Red confers on *A. brasilense* the ability to consistently spread in a semiliquid agar with formation of microcolonies. Spontaneous variants of *A. brasilense* with increased swarming rate are described, as well as variants that swarm in the presence of Congo Red. It is assumed that at least two types of compounds are formed, which are necessary for swarming and/or spreading with the formation of microcolonies and are capable of interacting with Congo Red.

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Key words: *Azospirillum brasilense*, colony morphology, motility, Congo Red.

Many microorganisms are capable of responding quickly to changes in the environment due to various locomotor organelles and modes of motion [1]. The bacterium *Azospirillum brasilense* interacts associatively with plants, swims in liquids (Mot^+ phenotype) using a single polar flagellum (Fla), and migrates collectively in viscous media using Fla and numerous lateral flagella (Laf) forming concentric swarming rings (Swa^+ phenotype). The production of Laf on *A. brasilense* cells is induced in media containing no less than 0.4% Bacto agar [2, 3]. *A. brasilense* Sp245 may also migrate in semiliquid media to form granular bacterial clusters, or microcolonies (Gri^+ phenotype; from granular inclusions). Presumably, the migration of the bacteria with the formation of microcolonies is determined by the activity of the polar bundle of pili, occasionally found on azospirillum cells instead of Fla [4]. In standard semiliquid media, ~0.3–1.0% of the clones with unstable Gri^+ phenotype and ~5–8% of immotile clones (Swa^- Gri^-) are found in the population of Sp245 along with the swarming bacteria [4]. In the case of Sp245 omegon Fla^-- or Fla^- Laf^- mutants that lost the ability to swarm, the number of clones with a pronounced Gri^+ phenotype reached 70–90% [4]. Insertion mutants of Sp245 with rapid swarming (superswarming) in semiliquid media (Swa^{++}) were also described [5]. The mechanisms underlying variations in the collective motility of bacteria, has colonial nature and depends not only on the function of locomotor organelles (flagella and pili), but also on the intercellular interactions [1], still needs to be studied in greater detail.

The motility of bacteria is influenced by alterations in the structure of their surface [1]. Presumably, adsorption of vital dyes on the cells may induce such alterations. For example, the carbohydrate-containing polymers of *A. brasilense*, represented by lipopolysaccharide–protein and polysaccharide–lipid complexes, as well as by polysaccharide mixtures, are capable of forming complexes with Congo Red (diphenyldiazo-*bis*-α-naphtylamine sulfonate) [6]. Consequently, the colonies of azospirilla become red on a solid medium containing the dye [7]. However, colonies colored pale pink, orange, or crimson appear in the populations of azospirilla with a frequency of up to ~1%; presumably, bacteria incapable of synthesizing one or more polysaccharides involved in dye adsorption form these colonies [7]. Note, however, that Congo Red may also interact with protein components of the bacterial surface [8].

The goal of this work was to study the effect of cell surface modifications (resulting from adsorption of Congo Red) on the behavior of *A. brasilense* and the generation of stable phenotypic variants of these bacteria with respect to collective motility.

MATERIALS AND METHODS

Azospirillum brasilense strains, Sp245 phenotypic variants, and the Sp245 mutants produced using pJFF350 (the vector for omegon mutagenesis [9]) used in the work are listed in Table 1.

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Table 1. *A. Brasilense* strains used in the work

A. brasilense strain	Characteristic	Reference or source
Sp7	Wild type isolated in Brazil from pangola grass rhizosphere	[2]
Cd	Wild type isolated in the USA from bermuda grass roots after inoculation with Sp7	[10]
Sp107	Wild type isolated in Brazil from wheat roots	[11]
S27	Wild type isolated in India from the bush *Sericostoma pauciflorum*	A.L. Lahiri, CAZRI, Jodhpur, India
SR8	Wild type isolated in Russia from smooth brome roots	[12]
SR15	Wild type isolated in Russia from orchard grass roots	[12]
SR55	Wild type isolated in Russia from wheat roots	[12]
SR75	Wild type isolated in Russia from wheat seedlings	[12]
SR80	Wild type isolated in Russia from wheat seedlings	L. Pozdnyakova, Institute of Biochemistry and Physiology of Plants and Microorganisms, Russian Academy of Sciences, Saratov
Sp245	Wild type isolated in Brazil from wheat roots	[13]
SK048	$Fla^{-}Mot^{-}Swa^{-}$ mutant of Sp245 (p120::Omegon-Km), Km^{R}	[14]
SK051	$Fla^{-}Laf^{-}Mot^{-}Swa^{-}$ mutant of Sp245 (p85::pJFF350), Km^{R}	[14]
SK248	$Mot^{-}Swa^{-}$ mutant of Sp245 (p85::pJFF350), Km^{R}	[14]
SK454	Fla^{-}leaky $Laf^{-}Mot^{-}Swa^{-}$ mutant of Sp245, Km^{R}	[14]
BK570	Swa^{++} mutant of Sp245 (p85::pJFF350), Km^{R}	[5]
Sp245.P1–Sp245.P5	Spontaneous Swa^{++} variants of Sp245	This paper
Sp245.CRP	Sp245 spontaneous variant swarming on semiliquid medium with Congo Red	This work
Sp245.P1.CRP1–Sp245.P1.CRP4	Sp245.P1 spontaneous variants swarming on semiliquid medium with Congo Red	This work

Azospirilla were cultivated on MSM (malate salt medium) [15]. The semiliquid MSM contained 0.2–0.6% Bacto agar. The adsorption of Congo Red by *A. brasilense* colonies was determined on a solid medium containing 37.5 μg/ml of the dye [7]. When needed, the medium was supplemented with 0.6–75 μg/ml Congo Red; pH values of all medium variants were adjusted to 6.8. The morphology of the zones of bacterial migration from the inoculation point into the semiliquid medium was assessed by the naked eye or using phase contrast microscopy. The mean speed of 50–100 individual bacterial cells was determined by computer analysis of video images (taken in observing the behavior of the bacteria) with the help of a Jenaval phase contrast microscope coupled to a Sony DCR-TRV900E video camera. The recording speed was 25 shots/s. The video files in *.avi format were stored on a PC and processed (using the program designed by V.A. Krestinenko) as follows: a cell was chosen using the mouse cursor, and the initial cell position was fixed by clicking the right mouse button to change the shot. Then the cell position was marked in Cartesian coordinates in a frame-by-frame mode. The movement trajectory was drawn in each frame to calculate the distance traversed and the average speed. The trajectory was stored as a dot image file; the distance covered and the average speed, as a text file.

At least five independent experiments (each run in triplicate) were performed in all cases. The results were statistically processed. The confidence intervals are given for a 95% significance level.

RESULTS AND DISCUSSION

Isolation of spontaneous variants of the model strain *A. brasilense* Sp245 displaying increased swarming rate in semiliquid media. According to our observations, the so-called "prominences" (or protrusions) with a higher swarming rate, coming from the outer boundary of the swarming rings, appeared rather frequently on days 3 and 4 after inoculation of Sp245 by injection into semiliquid MSM (Fig. 1a). Plating of prominences to individual colonies and rechecking of their swarming rates allowed us to select five spontaneous Sp245 derivatives with stable Swa^{++} phenotype (Sp245.P1–Sp245.P5). The diameter of swarming rings formed by Sp245.P1–Sp245.P5 exceeded the diameter characteristic of Sp245 approximately 2.4-fold. However, no more rapidly swarming clones appeared in the population of Swa^{++} derivatives of Sp245. The Swa^{++} phenotype was retained after reinoculation of the bac-

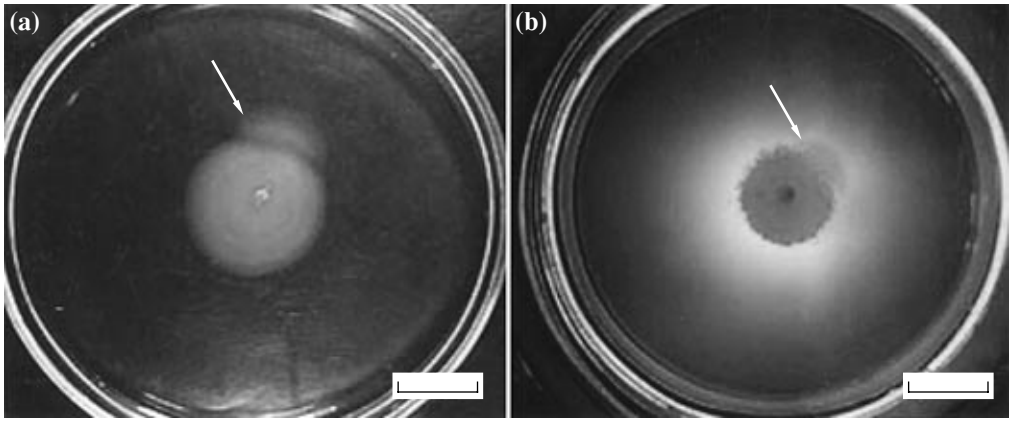

Fig. 1. Behavior of *A. brasilense* in MSM containing 0.4% agar: (a) formation of a swarming ring with a "prominence" (indicated with an arrow) in the dye-free medium (incubation time, 3 days) and (b) formation of a Gri^+ macrocolony and the outgoing swarming zone (indicated with an arrow) in the medium containing 37.5 μg/ml of Congo Red (incubation time, 7 days). The scale bar corresponds to 1 cm.

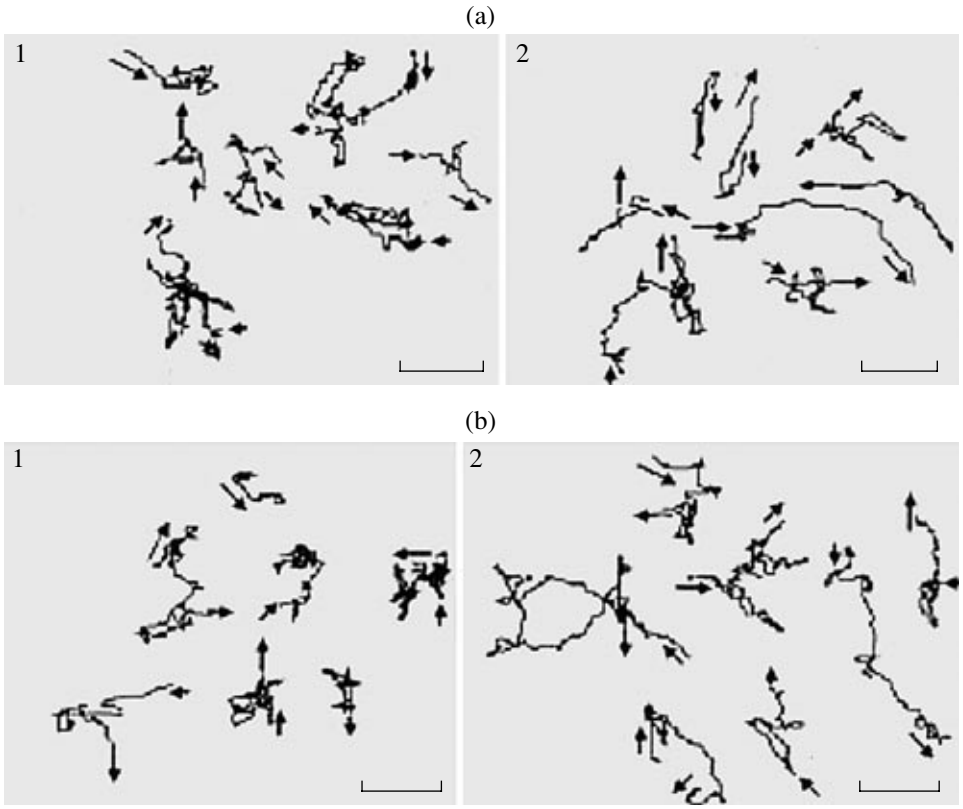

Fig. 2. Trajectories of individual cells of *A. brasilense* (1) Sp245 and (2) BK570 in MSM containing (a) 0.3% and (b) 0.4% agar. The start and end of trajectories are indicated with arrows. The scale bar corresponds to 10 μm.

teria on a dense or in a liquid MSM. The growth rates of *A. brasilense* Sp245 and its spontaneous superswarming derivatives in liquid MSM were practically identical. The colony morphology of Swa^{++} variants on a solid MSM was similar to that of the parental strain.

In liquid cultures, the cell movement pattern of Sp245 Swa^{++} derivatives did not differ from that of the wild type, whereas in the media containing 0.3–0.4% agar, the cells migrated to a greater distance than the wild-type cells (Fig. 2). In MSM supplemented with 0.3% agar, the speed of individual cells of Sp245 Swa^{++} derivatives (the cells carried Fla) was considerably higher than that of the original Sp245 cells; however, these distinctions disappeared at a higher agar concen-

Table 2. Mean migration speeds of cells of *A. brasilense* strain Sp245 and its derivatives, grown in liquid or semiliquid medium

A. brasilense strain	Mean cell migration speed, μm/sec				
	In liquid media		In semiliquid MSM with the agar concentration of		
	MSM	MSM + Congo Red*	0.3%	0.4%	0.6%
Sp245	29.0 ± 1.1	14.9 ± 0.7	14.4 ± 1.8	12.4 ± 1.1	8.7 ± 1.3
BK570	34.8 ± 1.0	21.4 ± 2.7	19.9 ± 1.8	14.0 ± 1.9	8.8 ± 1.2
Sp245.P1	36.8 ± 2.1	17.7 ± 1.0	17.7 ± 1.4	13.3 ± 0.6	8.2 ± 0.9
Sp245.P2	34.3 ± 1.9	18.9 ± 1.6	18.2 ± 1.2	14.3 ± 1.8	8.3 ± 1.3
Sp245.P3	36.0 ± 2.2	22.4 ± 2.3	18.1 ± 1.3	14.1 ± 1.2	8.7 ± 1.4
Sp245.P4	36.7 ± 3.4	19.8 ± 2.0	17.3 ± 1.2	14.0 ± 1.3	8.4 ± 1.2
Sp245.P5	35.3 ± 2.5	19.2 ± 2.2	18.2 ± 1.8	14.2 ± 1.6	8.6 ± 1.3
Sp245.CRP	28.5 ± 2.0	26.0 ± 1.8	13.6 ± 1.3	12.8 ± 1.1	8.2 ± 1.1
Sp245.P1.CRP1	30.5 ± 2.9	26.7 ± 1.9	13.6 ± 1.5	11.8 ± 1.8	7.8 ± 1.0
Sp245.P1.CRP2	29.3 ± 2.3	24.5 ± 1.9	13.7 ± 1.2	12.6 ± 1.0	8.7 ± 1.0

* The concentration of Congo Red was 37.5 μg/ml.

tration (the cells carried both Fla and Laf; Table 2). It would seem that both the extension of the linear motion trajectory of Swa^{++} strain cells and the increase in their speed depended on the activity of the polar flagellum.

Motilities of *A. brasilense* Sp245, its spontaneous variants, and omegon mutants in the presence of Congo Red. To determine the ability of *A. brasilense* strain Sp245, its Swa^{++} derivatives, and the mutants incapable of swarming to adsorb Congo Red, the bacteria were plated onto the solid medium containing 37.5 μg/ml of the dye, as described in [7]. Unlike *A. brasilense* strains Sp245, BK570, Sp245.P1, Sp245.P2, and Sp245.P3, which formed ruby colonies on solid MSM containing Congo Red, the colonies of Sp245.P4, Sp245.P5, SK048, SK051, SK248, and SK454 were pale pink. Thus, we failed to find a strict correlation between the intensity of dye adsorption by the bacteria grown on solid medium, on the one hand, and the colony size and morphology in semiliquid MSM, on the other.

CFU (colony-forming unit) counts in liquid 24-h-old cultures of *A. brasilense* Sp245 (in the dye-free MSM and MSM with 37.5 μg/ml Congo Red) were nearly equal (8.1×10^7 and 8.3×10^7 CFU, respectively). CFU counts were independent of the type of agar medium (conventional MSM or MSM supplemented with Congo Red) used for plating serial dilutions of liquid azospirillum cultures. The azospirillum cells grown in liquid MSM containing 37.5 μg/ml of the dye possessed a normal polar flagellum. When cultivating azospirilla in liquid MSM with Congo Red (but not adding the dye to the culture already grown), the speed of azospirillum cell migration decreased (except for CRP cells; see Table 2 below).

In the semiliquid MSM containing 37.5 μg/ml Congo Red, the strains *A. brasilense* Sp245, Sp245.P1–Sp245.P5, and BK570 changed their behavior drastically, commencing migration to form microcolonies (Fig. 1b). The macrocolonies were colored light red.

A. brasilense Sp245 and its Swa^{++} derivatives started migrating in the semiliquid MSM with Congo Red within 24 h (vs. 2.5–5 h in the dye-free MSM) of inoculation. In the case of the mutants SK048, SK051, SK248, and SK454, Congo Red had no effect on the duration of the stage preceding the spreading of bacteria and microcolony formation (~42 h).

The derivatives of Sp245 (Sp245.CRP; Fig. 1b) and Sp245.P1 (Sp245.P1.CRP1–Sp245.P1.CRP4), which were capable of spreading by swarming in the presence of the dye, were isolated. Unlike the light red Gri^+ macrocolonies, their swarming zones acquired orange color in the semiliquid medium. On a solid MSM, the CRP variants adsorbed Congo Red in different ways. For example, the colonies of Sp245.CRP and Sp245.P1.CRP1 were pale pink, while the colonies of Sp245.P1.CRP2 had a ruby color. In other words, changes in the density of culture media, concentration of the available oxygen, and/or local concentration of bacteria influenced the structure of the cell surface of azospirillum that interacted with the dye.

The diameters of swarming rings formed by the strains Sp245.P1.CRP(1–4) and Sp245.CRP in semiliquid MSM were approximately 32% smaller that of Sp245. In the presence of 37.5 μg/ml Congo Red, the diameters of swarming rings of Sp245.CRP and Sp245.P1.CRP(1–4) decreased by 30%; the diameter of the granular macrocolonies of Sp245 was one half as large as that of the swarming rings of CRP clones.

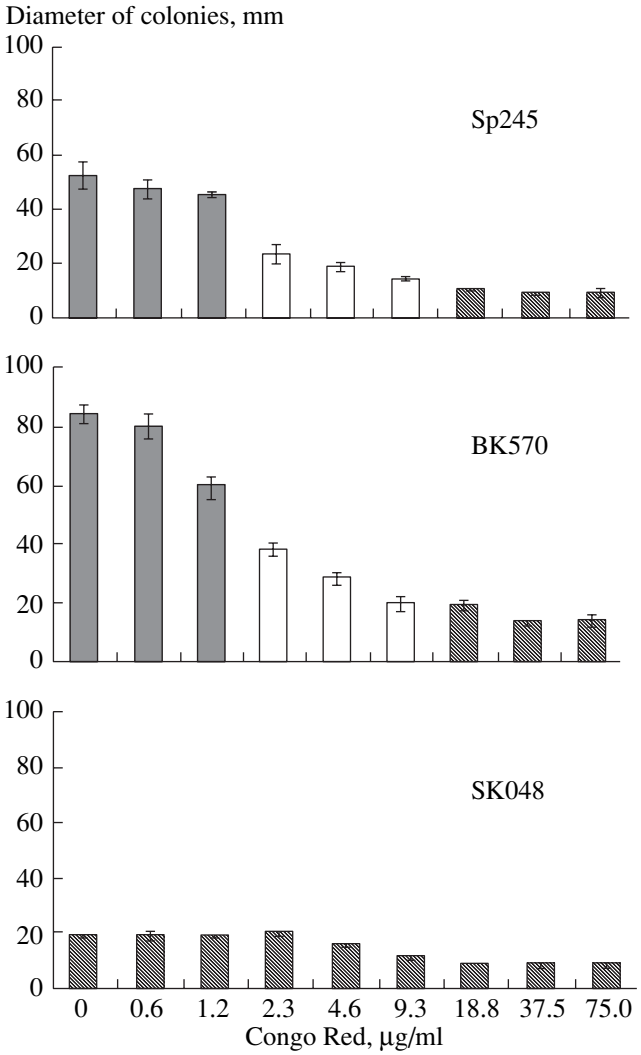

Fig. 3. Effect of Congo Red on the size and morphology of macrocolonies formed by *A. brasilense* Sp245 and its omegon mutants BK570 Swa^{++} and SK048 Fla^{-} Mot^{-} Swa^{-} over 96 h in MSM containing 0.4% agar. Formation of concentric swarming rings is denoted by dark rectangles; of turbid granular colonies, by light rectangles; and of granular colonies, by light hatched rectangles.

Sp245, Sp245.CRP, and Sp245.P1.CRP cells migrated in the dye-free MSM at about the same speeds (Table 2). Sp245.CRP and Sp245.P1.CRP cells grown in the liquid medium supplemented with Congo Red retained the migration speed almost completely, unlike the wild-type strain and its superswarming mutants (Table 2). Presumably, the variants Sp245.CRP and Sp245.P1.CRP are capable of surmounting the inhibitory action of Congo Red due to a modification of extracellular compounds that form complexes with the dye.

The effect of various Congo Red concentrations on the collective motility of *A. brasilense* Sp245 and its mutants in semiliquid MSM was studied (Figs. 3 and 4). It was found that Sp245 and BK570 switched from forming concentric swarming rings (at a concentration of Congo Red not exceeding 2.3 μg/ml) through macrocolonies of mixed turbid-granular phenotype (in the presence of 2.3–9.3 μg/ml Congo Red) to spreading with eventual formation of microcolonies (at a concentration of the dye in excess of 9.3 μg/ml; Fig. 3). Phase contrast microscopy demonstrated that the granular macrocolonies comprised cell clusters located close to one another (Fig. 4a–4c). On the media containing Congo Red, the collective motility of SK048 decreased somewhat (Fig. 3), but the granular microcolony morphology was preserved (Fig. 4a–4c). The behavior of the mutants SK051, SK248, and SK454 did not differ from that of SK048 (Fig. 4a, 4b). Thus, Congo Red affected the speed of collective migration of all the strains studied and the morphology of macrocolonies formed by the strain Sp245 and its Swa^{++} derivatives.

The mean migration speeds of single Sp245 cells grown in the liquid MSM with Congo Red or in the semiliquid MSM containing 0.3–0.4% agar differed insignificantly (Table 2). In the semiliquid media without Congo Red, Sp245 cultures formed concentric swarming rings regardless of agar concentration (in the range of 0.3–0.6%) and the degree of cell motility suppression (Table 2). The granular Sp245 macrocolonies were formed only in the presence of the dye.

Presumably, *A. brasilense* Sp245 produces at least two compounds that interact with Congo Red and facilitate collective bacterial migration. Once Congo Red blocks a compound necessary for cell swarming, the motility of bacteria decreases and the Gri^{+} phenotype is induced. The other compound, likely to form a less stable or specific complex with the dye, may be necessary for both the swarming and the migration of azospirilla, with the formation of microcolonies. A weak interaction of Congo Red with the second compound produces a small decrease in the Gri^{+} spreading speed of bacteria. When the first compound is blocked, the other components of the cell surface may compensate for the missing functions (for example, through increasing the production level), assist cells in surmounting the inhibiting effect of Congo Red, and restore the swarming (unless the flagellar machinery of the cell is damaged by a mutation).

The effect of Congo Red on the motility of wild-type *A. brasilense* strains isolated in different countries. The bacteria of the genus *Azospirillum* are capable of interacting associatively with an extremely broad range of plant species. When colonizing plant roots, azospirilla frequently form microcolonies; however, the underlying mechanisms are yet unknown [16]. Thus, it was interesting to determine the degree to which the adsorption of Congo Red (possibly mimicking effects of certain root surface components) influenced the behavior of azospirilla. Ten wild-type *A. brasilense* strains formed granular colonies on the medium containing Congo Red, irrespective of their origin or swarming rate on the dye-free medium (Tables 1 and 3).

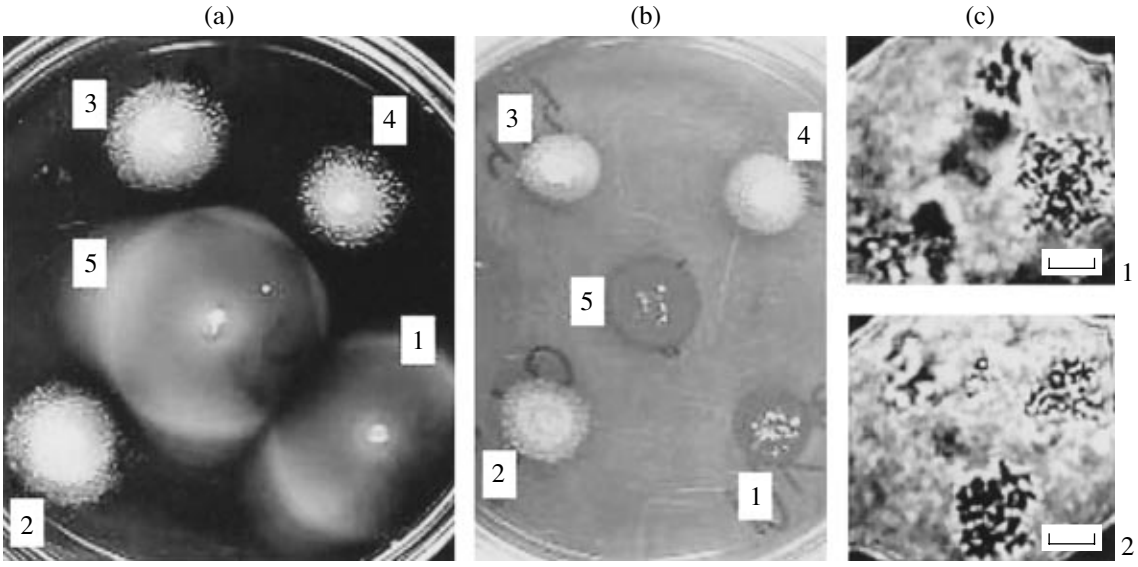

Fig. 4. Colonies formed by (1) *A. brasilense* Sp245, and the mutants (2) SK048 Fla⁻ Mot⁻ Swa⁻, (3) SK454 Fla⁻ leaky Laf⁻ Mot⁻ Swa⁻, (4) SK051 Fla⁻ Laf⁻ Mot⁻ Swa⁻, and (5) BK570 Swa^{++} over 120 h in MSM containing 0.4% agar: (a) without Congo Red, (b) in the presence of Congo Red (37.5 μg/ml), and (c) phase contrast microscopy of macrocolonies 1 and 2 from panel (b). The scale bar in panel (c) corresponds to 10 μm.

In the available literature, we found only one paper reporting the effect of Congo Red on the microbial behavior. The dye binds to the cell surface of a soil bacterium, *Myxococcus xanthus*, inhibiting agglutination, social motility, and the formation of fruiting bodies [17]. The main receptor for Congo Red is presumably localized on fibrils of myxobacteria (long polysaccharide filaments associated with a multimeric protein) [17].

In this work, we have demonstrated for the first time that adsorption of Congo Red on cells may lead to (1) inhibition of bacterial swarming and (2) consistent manifestation of the ability to spread in a semiliquid medium with the formation of microcolonies. The emergence of spontaneous *A. brasilense* variants capable of swarming rapidly or swarming in semiliquid media in the presence of Congo Red was also described for the first time. The data obtained demonstrate that *A. brasilense* has several systems of collective motility, which ensure bacterial migration under diverse conditions.

Table 3. Motility of wild-type *A. brasilense* strains under different conditions

A. brasilense strain	18-h-old culture in liquid MSM without Congo Red		Size of macrocolonies (mm)* in MSM + 0.4% agar	
	Motile cell count, %	Cell migration speed, μm/sec	Without Congo Red**	With Congo Red (37.5 μg/ml)***
Sp7	85.5 ± 2.2	36.7 ± 2.8	14.0 ± 2.8	7.2 ± 0.8
Cd	87.3 ± 2.6	34.6 ± 3.1	30.3 ± 5.9	10.5 ± 0.9
Sp107	87.0 ± 2.2	34.3 ± 5.2	35.4 ± 6.5	11.0 ± 1.8
Sp245	85.8 ± 3.8	29.0 ± 1.1	23.8 ± 2.5	10.0 ± 1.2
S27	85.2 ± 1.0	30.6 ± 2.0	11.3 ± 3.3	9.0 ± 1.8
SR8	85.2 ± 2.2	30.5 ± 3.4	24.3 ± 2.0	8.6 ± 1.9
SR15	86.6 ± 3.8	27.8 ± 3.4	36.9 ± 5.8	9.0 ± 1.5
SR55	58.3 ± 9.4	27.1 ± 2.4	17.3 ± 2.4	7.4 ± 0.7
SR75	60.9 ± 5.4	30.3 ± 3.3	19.0 ± 5.0	7.9 ± 1.8
SR80	51.6 ± 9.0	23.1 ± 2.4	15.3 ± 3.9	6.4 ± 0.8

* Diameter of inoculation point, 4.5 ± 0.3 mm; incubation time, 48 h.
** Swarming rings.
*** Granular macrocolonies.

ACKNOWLEDGMENTS

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