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Isolation of Bacterial Strains Capable of Sulfamethoxazole Mineralization from an Acclimated Membrane Bioreactor

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In this study, we isolated five strains capable of degrading ^{14}C-labeled sulfamethoxazole to $^{14}CO_2$ from a membrane bioreactor acclimatized to sulfamethoxazole, carbamazepine, and diclofenac. Of these strains, two belonged to the phylum *Actinobacteria*, while three were members of the *Proteobacteria*.

The antibiotic sulfamethoxazole (SMX) undergoes only partial removal in municipal wastewater treatment plants (WWTP) and is often detected in treated effluents and receiving water bodies at concentrations up to 1.9 μg/liter (6, 8). It is suspected to promote the development of antibacterial resistance in water bodies (3). Little information on the bacterial and fungal transformation/degradation of SMX is available. The versatile peroxidase of *Bjerkandera adusta* was demonstrated to transform SMX to 3-amino-5-methylisoxazole and a range of oxidation products (4). Previous studies reported the transformation of SMX by bacteria from microorganism collections. *Rhodococcus rhodochrous* and *Pseudomonas aeruginosa* were shown to cometabolize SMX (5, 7). Only *Rhodococcus equi* led to the transformation of SMX without glucose as a cosubstrate (7). Among the tentatively identified biotransformation products were a deamination product (5) and acetyl and hydroxyacetyl conjugates of SMX. Nevertheless, until now, no evidence for mineralization of SMX by axenic strains has been reported. This is especially important because removal of SMX can be biased by choosing unfavorable sampling conditions.

In the present study, five strains capable of SMX mineralization were isolated from a lab-scale membrane bioreactor (MBR) acclimatized with a synthetic effluent loaded with pharmaceuticals.

The 1.5-liter MBR was operated continuously as described in reference 1. The reactor was fed with a complex medium adapted from DIN ISO 11733 (2) and spiked with SMX, carbamazepine, and diclofenac to a final concentration of 100 μg/liter (each). It was operated under steady conditions for 10 months before sampling of the biomass to inoculate enrichment cultures. The hydraulic retention time was 12 h, the sludge retention time was infinite, and the total concentration of solids was 6 g/liter, on average. At the time of biomass sampling, the average SMX removal rate in the MBR was 52% (data not shown).

Enrichment cultures were prepared in Erlenmeyer flasks containing 100 ml of mineral salts medium (10) containing 0.5 mM SMX as the sole carbon source (MSM-S) and were inoculated with 2-ml samples of biomass from the MBR before incubation at 28°C on a rotary shaker at 130 rpm. Twice, after a 1-month incubation each time, half of the culture volume was replaced with fresh enriched MSM-S. After another month, 3 ml of enrichment culture was used to inoculate 100 ml of fresh MSM-S. A month later, the SMX-degrading enrichment culture was diluted in 0.85% (wt/vol) NaCl and plated on plate count agar (PCA) (medium 464; DSMZ, Germany) and, to possibly select for isolated fungi, Sabouraud agar (Sigma-Aldrich, Switzerland). Plates were incubated overnight at 28°C, and six morphologically distinct types of colonies could be isolated. Two colonies of each type were picked and isolated on PCA plates.

Mineralization experiments were carried out in Erlenmeyer flasks containing 20 ml of fresh MSM-S containing [^{14}C]SMX ([^{14}C]aniline [uniform]; Hartmann Analytic, Germany) with a specific radioactivity of 0.33 MBq/mmol. Two-milliliter centrifugation tubes filled with 1 ml of 5 M NaOH were fixed by copper wire in the headspace of the flasks to trap formed $^{14}CO_2$. Flasks were sealed with an airtight rubber plug and wrapped in aluminum foil to prevent photodegradation. They were incubated at 28°C on a rotary shaker at 130 rpm. Trapped CO_2 was precipitated as $BaCO_3$ by adding 5 volumes of 1 M $BaCl_2$. After centrifugation at 1,000 × *g* for 10 min, precipitates were resuspended in liquid scintillation cocktail (Irgasafe Plus; PerkinElmer, Switzerland) and analyzed in a liquid scintillation counter (Tri-Carb 2800TR; PerkinElmer, Switzerland). The radioactivity recovered in the NaOH traps was used to calculate the amount of mineralization. At the end of each experiment, remaining radioactivity in the medium was determined. Radioactivity balances were between 89 and 109% for all samples.

For mineralization experiments with the enrichment cultures, 1 ml of culture obtained in the last step of the enrichment series was added to the Erlenmeyer flasks. After 8 days of incubation, the first signs of mineralization were observed, when an average of 0.3% ± 0.05% ($n = 3$) of the total applied radioactivity was recovered in the 5 M NaOH solution. The percentage of radioactivity recovered as $^{14}CO_2$ increased gradually, to reach a plateau at 58.0% ± 1.3% after 24 days of incubation (Fig. 1a).

For mineralization of SMX by the isolated strains, the latter were grown on PCA plates for 48 h at 28°C and suspended in sterile physiological water (0.85% [wt/vol] NaCl) to a final optical density at 600 nm (OD_{600}) of 1.0, after which 500 μl was used to

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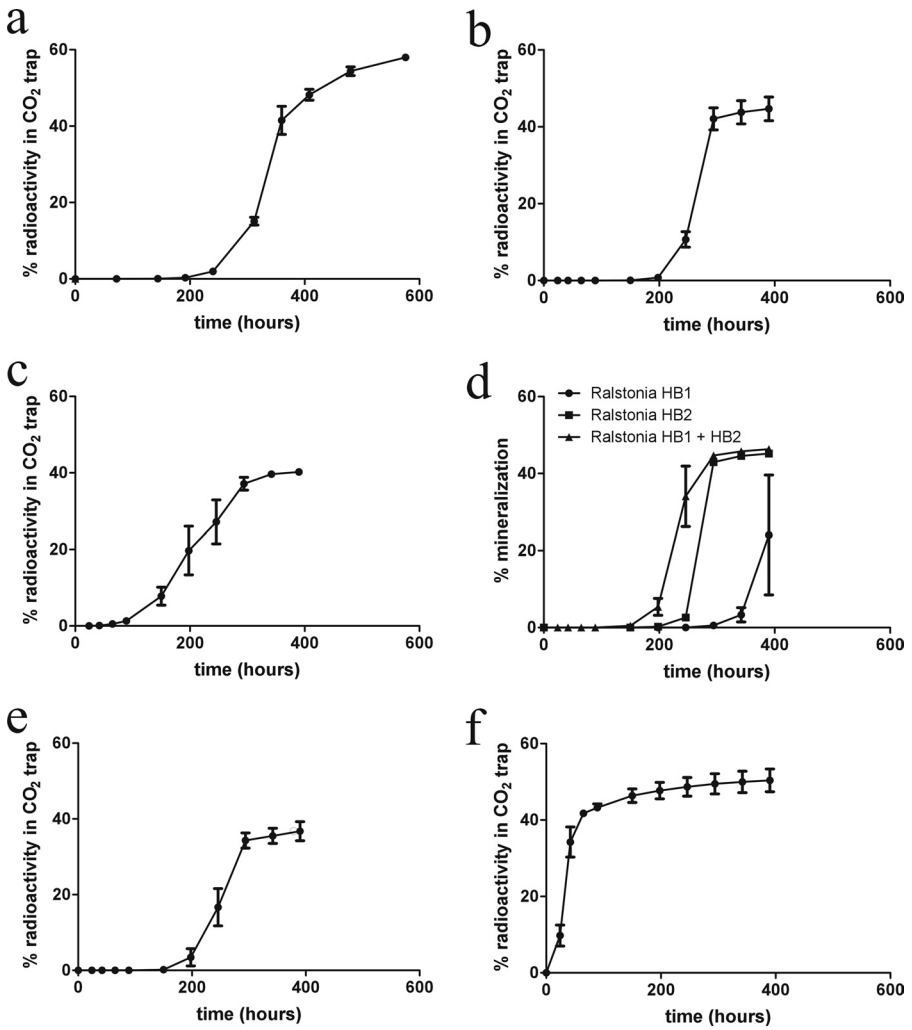

FIG 1 Mineralization of sulfamethoxazole in mineral medium enriched with 0.5 mM ^{14}C-labeled sulfamethoxazole. (a) Enrichment culture; (b) *Achromobacter* sp.; (c) *Microbacterium* sp.; (d) *Ralstonia* sp.; (e) *Rhodococcus* sp.; (f) *Microbacterium* sp. and *Rhodococcus* sp.

inoculate the Erlenmeyer flasks. The analysis of the degradation tests revealed that five of six isolates could mineralize SMX, as 24 to 44% of the total radioactivity was found in the respective CO_2 traps after 16 days (Fig. 1b through f). Neither the abiotic control nor the samples containing the *Tsukumurella* sp. HB3 isolate showed any decrease of radioactivity in the liquid phase or an accumulation of $^{14}CO_2$ (data not shown).

Fragments of 16S rRNA genes of the isolates mineralizing SMX were amplified by colony PCR with universal 16S rRNA gene primers B27f and U1492r (9). Amplified fragments were sequenced by Eurofins MWG Operon (Germany), using 336r and 928f (11) or B27f and U1492r as sequencing primers. Obtained sequences were subjected to nucleotide BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

The identified strains belong to the phyla *Actinobacteria* (*Microbacterium* sp. strain BR1 and *Rhodococcus* sp. strain BR2) and *Proteobacteria* (*Achromobacter* sp. strain BR3 and *Ralstonia* sp. strains HB1 and HB2) (Table 1). Although both *Ralstonia* strains were found to have identical 16S rRNA genes, they possessed different morphologies on PCA plates, as strain HB1 formed small yellow colonies and strain HB2 formed larger, off-white colonies.

We investigated mineralization in an SMX-degrading enrichment culture from which we later isolated five strains able to mineralize SMX. This is, to our knowledge, the first report of such high SMX degradation and actual proof of mineralization both in enriched cultures and in axenic strains. Interestingly, cocultures of *Microbacterium* sp. BR1 and *Rhodococcus* sp. BR2 showed both

TABLE 1 Strains isolated from the acclimatized MBR and identification by 16S rRNA gene sequencing

Isolate	Identification	Phylum	GenBank accession no.
BR1	*Microbacterium* sp.	*Actinobacteria*	JN196543
BR2	*Rhodococcus* sp.	*Actinobacteria*	JN196542
BR3	*Achromobacter* sp.	*Proteobacteria*	JN196540 and JN196541[a]
HB1	*Ralstonia* sp.	*Proteobacteria*	JN196539
HB2	*Ralstonia* sp.	*Proteobacteria*	JN196538
HB3[b]	*Tsukumurella* sp.	*Actinobacteria*	JN851820

[a] Two sequences were obtained, differing by one base pair.
[b] No mineralization of SMX was observed in cultures containing this strain.

higher mineralization rates and shorter lag times than cultures of the respective single strains, probably caused by syntrophy. To a lesser extent, the same could be observed for samples containing a mixture of both *Ralstonia* isolates.

Currently, experiments are being carried out to investigate possible accumulating metabolites.

Nucleotide sequence accession numbers. The nucleotide sequences obtained in this study are available in GenBank under the accession numbers listed in Table 1.

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