

Biodegradation of sulfamethoxazole and other sulfonamides by *Achromobacter denitrificans* PR1

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Abstract

This study aimed to isolate and characterize a microbial culture able to degrade sulfonamides. Sulfamethoxazole (SMX)-degrading microorganisms were enriched from activated sludge and wastewater. The resultant mixed culture was composed of four bacterial strains, out of which only *Achromobacter denitrificans* PR1 could degrade SMX. This sulfonamide was used as sole source of carbon, nitrogen and energy with stoichiometric accumulation of 3-amino-5-methylisoxazole. Strain PR1 was able to remove SMX at a rate of $73.6 \pm 9.6 \mu\text{mol SMX/g cell dry weight h}$. This rate more than doubled when a supplement of amino acids or the other members of the mixed culture were added. Besides SMX, strain PR1 was able to degrade other sulfonamides with anti-microbial activity. Other environmental *Achromobacter* spp. could not degrade SMX, suggesting that this property is not broadly distributed in members of this genus. Further studies are needed to shed additional light on the genetics and enzymology of this process.

1. Introduction

Sulfonamides are synthetic drugs used as anti-microbial, anti-diabetic, diuretic, anticonvulsant, and herbicidal agents [1]. Consequently, sulfonamides are pollutants widely distributed in domestic wastewaters [2]. Sulfamethoxazole (SMX) is one of the most prescribed antibiotics worldwide. Given that more than 50% of the ingested SMX may be excreted without modification [3], it can reach concentrations up to 7910 ng/L in wastewater treatment plants [4]. Depending

on the wastewater treatment process and other external conditions, including climate, SMX removal can vary between 0 and 100% [2]. Therefore, the contamination of other water bodies is unavoidable, and SMX has been detected in concentrations up to 1900 and 1110 ng/L in surface and groundwater, respectively [5,6]. Adverse effects of SMX environmental contamination have been demonstrated in algae and fish hepatocytes [7,8]. Additionally, SMX undergoes photodegradation, which may result in the formation of products with increased toxicity [9]. Besides the potential adverse effects in plants, animals or microorganisms, the influence of this contaminant on the selection of antibiotic resistant bacteria cannot be discarded. Indeed, the occurrence of sulfonamide residues, including those of SMX, in river water correlated positively with the presence of sulfonamide resistance genetic determinants [10]. Sulfonamide resistance in water habitats can reach values up to 94% [11], being observed in many commensal and pathogenic bacteria, in particular multidrug resistant [12]. Moreover, sulfonamide resistance genes, often associated with mobile genetic elements, have a widespread distribution in the environment [13]. For these reasons, sulfonamides are relevant environmental contaminants, whose removal from waters requires efficient and sustainable mitigation measures.

The removal of anti-microbial drugs from wastewater is a challenging issue. Since conventional processes were not designed for that purpose, micro-pollutants are not efficiently removed during wastewater treatment. Processes such as those based on advanced oxidation processes, adsorption to activated carbon or membrane reactors can efficiently remove different organic micro-pollutants from waters, including SMX [2]. However, for technical and economic reasons, the implementation of these processes may not be feasible in some urban wastewater treatment plants [2]. The removal of xenobiotic compounds via biological processes has several advantages, being regarded as a green technology, with minimal environmental impacts and low operational costs. Most studies on SMX biodegradation assessed its removal by activated sludge [14–18] and only few studies identified the bacterial strains involved in the process. The first examples were reported for members of the genus *Rhodococcus*, which were able to co-metabolize SMX with glucose in axenic culture [19,20]. Later, other organisms, all enriched from activated sludge, belonging to the genera *Achromobacter*, *Ralstonia*, *Pseudomonas*, *Brevundimonas*, *Variovorax*, *Rhodococcus*, and *Microbacterium* were reported as capable of degrading SMX [21–23]. The diversity and distribution of SMX biodegradation in wastewater environments is still a major question, since this property has been detected in distantly related phylogenetic lineages and geographic regions. Besides the interesting issues raised by SMX biodegradation, which comprise the evolution of degradation pathways or the resistance development, this property may also have a practical application. Indeed, SMX-degrading bacteria or enzymes thereof may be potentially used as low cost biotreatment tools, as long as measures to

avoid antibiotic resistance spread can be assured. Although wastewater environments comprise complex mixtures of micro-pollutants, studies focusing the biodegradation of individual compounds by microbial cultures are fundamental to understanding their biological evolution and potential application. With this rationale in mind, this study aimed to isolate bacteria able to degrade SMX from wastewater environments and to characterize the degradation process in terms of rate, transformation products, and optimal metabolic conditions for degradation.

2. Material and methods

2.1. Chemicals

Reagent grade (purity $\geq 99.0\%$) sulfamethoxazole, sulfanilic acid, sulfadimethoxine, sulfapyridine, and sulfamethoxypyridazine were from Fluka. Glimepiride, gliclazide, glibenclamide and furosemide were from Sigma–Aldrich. Sulfadiazine, sulfathiazole, sulfacetamide sodium salt dihydrate were from Acofarma. β -Nicotinamide adenine dinucleotide (NADH) disodium salt trihydrate, and 3-amino-5-methylisoxazole (3A5MI) were from Merck. Sulfamethazine was from Alfa Aesar. [Ring- U - ^{14}C]aniline-SMX with a specific radioactivity of 0.33 MBq/mmol was from Hartmann Analytic. All other reagents were of analytical grade from commercial sources.

2.2. Growth media and culture conditions

The cultures were grown in mineral medium B [24] with 0.5 g/L ammonium sulfate as nitrogen source (herein designated MMBN) and supplemented with SMX at different concentrations. The use of SMX as the single source of carbon, nitrogen, and energy was assessed in cultures grown in MMBN without ammonium sulfate. The effect of additional sources of carbon and energy or micronutrients was tested in MMBN supplemented with succinate or a mixture of amino acids alone or in combination with vitamins and nitrogenous bases [25]. Agar media were brain–heart Infusion agar diluted four times (Oxoid, Basingstoke, England) supplemented with 0.15 g/L SMX (0.6 mM) (25% BHI-SMX) or MMBN with agar (17 g/L) supplemented with 1.3 g/L SMX (5 mM) as sole carbon and energy source (MMBN-SMX agar). All cultures were incubated at 30 °C in darkness and broth cultures with orbital shaking at 120 rpm. Unless mentioned otherwise, the initial cell density ranged from 0.026 to 0.041 gbiomass/L (1 unit of optical density at 600 nm corresponded to 0.526 gbiomass/L).

2.3. Enrichment cultures and isolation of bacteria

Samples of activated sludge and treated domestic wastewater collected from a wastewater treatment plant in the North of Portugal were inoculated in MMBN (50%, v/v) with 0.25 g/L SMX and 1 g/L yeast extract (YE). These cultures, designated 7 and 8, were transferred weekly to fresh medium (10%, vinoculum/vculture) with increasing SMX and decreasing YE content (up to 0.32 g/L and down to 0.70 g/L, respectively). After 7 months, cultures were pooled, into culture 78, and grown in MMBN with 0.12 g/L SMX and 1.2 g/L succinate. After three successive transfers, culture 78 was serially diluted in sterile saline solution (0.85%, w/v) and plated on 25% BHI-SMX. After 48 h of incubation, all the biomass grown on the dilution plates (10^{-1} – 10^{-7}) was suspended in sterile saline solution and transferred to MMBN supplemented with 0.15 g/L (0.6 mM) SMX and 0.6 g/L (5 mM) succinate. The culture corresponding to the most diluted fraction of the enrichment 78 that maintained the capacity to degrade SMX (10^{-2}) was designated C78 and preserved for further studies (Fig. 1).

Culture C78 was subjected to serial dilution limit as described elsewhere [26]. Briefly, it was serially diluted and plated on MMBN- SMX agar and incubated for 20 days. The resultant biomass grown on each dilution plate was suspended and transferred to MMBN with 0.6 mM SMX and the dilution-cultivation procedure was repeated (Fig. 1). Twenty-six colonies were isolated on 25% BHI- SMX, which comprised culture C78S (Fig. 1). All cultures were stored at -80°C in a mixture of 15% glycerol and MMBN.

2.4. Composition of the mixed cultures and identification of the isolates

Total DNA was extracted from 0.76 mg of biomass of mixed cultures 78, C78, and C78S using a commercial kit (PowerSoilTM DNA Isolation Kit, MO BIO, Carlsbad, CA) [27]. Crude cell lysates of the 26 isolates were obtained after boiling (5 min). Mixed cultures were compared by denaturing gradient gel electrophoresis (DGGE), based on the V3 region of the 16S rRNA gene [27]. About 1.2 μg DNA was loaded in a 8% (w/v) polyacrylamide gel with a 25–60% denaturant gradient (100% denaturant contained 7 M urea and 40% (v/v) formamide). DGGE was run for 5.5 h at 200 V and 60°C . Relevant isolates were identified based on the 16S rRNA gene sequence analysis (amplicon size > 1200 bp), and the EzTaxone server (<http://www.ezbiocloud.net/eztaxon>). The 16S rRNA gene sequences of strains PR1, PR2, PR3, and PR4 were deposited in GenBank with the accession numbers KJ124851, KJ124852, KJ124853, and KJ124854, respectively.

2.5. Mineralization assays

To assess SMX mineralization, i.e., the transformation of SMX to CO₂, a suspension of mixed culture C78 at an initial density of 0.013 g cell dry weight/L was incubated in MMBN with 0.6 mM ¹⁴C-SMX ([Ring-U-¹⁴C]aniline-SMX), as sole carbon source. Radioactivity was determined according to Bouju et al. [21]. The radioactivity remaining in the biomass and in the culture supernatant was determined after centrifugation at 20,000 *g* for 15 min and filtration (Titan2, 0.45 μm).

2.6. SMX degradation by mixed culture C78S or its members

The ability of mixed culture C78S to use SMX as single source of carbon and energy was tested in MMBN supplemented with 0.6, 5, and 10 mM SMX (0.15, 1.3, and 2.5 gSMX/L, corresponding, respectively, to 0.07, 0.60, and 1.20 g carbon/L) in Teflon lined capped Erlenmeyer flasks. Cells growth and SMX depletion were monitored up to 7, 103, and 188 days.

Possible retro-inhibition by 3A5MI on SMX degradation was assessed in C78S cultures grown MMBN with 0.6 mM SMX and 20 mM 3A5MI over 6 days of incubation. Anaerobic degradation of SMX by culture C78S was assayed in MMBN with 0.6 mM SMX, 5 mM succinate, and 10 mM KNO₃ as an electron acceptor, under a nitrogen atmosphere in PTFE-sealed flasks, incubated for 31 days.

To assess abiotic losses and/or adsorption of SMX onto cells, all assays included controls without inoculum and with autoclaved biomass, respectively.

SMX degradation by pure isolates of culture C78S was tested in MMBN with 0.6 mM SMX or with SMX and succinate, or growth factors (amino acids alone or together with vitamins and nitrogenous bases). Cultures and abiotic controls were incubated up to 14 days.

2.7. SMX degradation assays by crude cell extracts

Crude cell extracts were prepared from a cell suspension of strain PR1 (OD₆₀₀ nm equivalent to 30) in saline phosphate buffer (PBS), using biomass from a culture pre-grown in MMBN with 0.6 mM SMX and 5 mM succinate. The capacity of this suspension to degrade SMX (positive control), was tested in a resting cells assay at 30 °C in PBS with 0.6 mM SMX and an initial OD₆₀₀ nm of 10 (corresponding to approximately 2.4 g protein/L). Crude cell extracts were prepared by disruption by sonication (UP50H-Hielscher, at 80% amplitude 50/60 Hz, 0.5 cycles for 1 min) in an ice/salt bath, repeated 3 times. The extract was centrifuged (20,000 × *g*, 15 min, 4 °C) and the total protein content in the supernatant was

determined using the Bradford reagent (Sigma–Aldrich, Germany). The degrading capacity of the crude cell-free extracts (corresponding to approximately 6.7 0.3 gprotein/L) was assayed in PBS with 0.1 mM SMX. The NADH requirement for SMX degradation was tested using coenzyme concentrations from 1 to 4 mM [28]. The reaction mixtures were incubated at 30 °C in the dark up to 13 h. Prior to SMX analysis, proteins were removed by precipitation with 2% (v/v) HCl at room temperature for 10 min, and centrifugation at $20,000 \times g$ for 15 min.

2.8. Degradation of othersulfonamides

The ability of strain PR1 to degrade sulfapyridine, sulfamethazine, sulfamethoxypyridazine, sulfadiazine, sulfathiazole and sulfadimethoxine (anti-microbials), glibenclamide, glimepiride, gliclazide (hypoglycaemic agents), furosemides (diuretic agent), and sulfacetamide (an acne treating drug) was carried out in MMBN supplemented with amino acids vitamins and nitrogenous bases and with 0.1 mM of each compound. Abiotic controls were incubated in parallel.

2.9. Analyticalprocedures

Cell growth was followed by spectrophotometry (OD600nm, Unicam Helios spectrophotometer), based on calibration curves of optical density versus cell dry weight [24]. The concentra- tion of tested sulfonamides was determined by HPLC–UV–vis (Knauer) with a 5 µm Purospher®STAR 100 RP-18e (Merck). SMX and furosemide were determined at 270 nm. A mixture of 20 mM $\text{NH}_4\text{H}_2\text{PO}_4$ and acetonitrile (70:30, v/v) acidified at pH 2.8 with oxalic acid was used at 1 mL/min. Gliclazide, glimepiride, glibenclamide were detected at 230 nm with a mobile phase composed by methanol and 20 mM KH_2PO_4 at pH 4 (70:30, v/v). The detection of sulfadimethoxine was carried out with a mixture of methanol and water (60:40, v/v) and the wavelength was set to 250 nm. Sulfapyridine, sulfamethazine, sulfamethoxypyridazine, sulfadiazine, sulfathiazole, and sulfacetamide were detected at 280 nm, with a mixture of 22 mM formic acid and methanol (75:25, v/v). 3A5MI was determined at 230 nm, using a mixture of 22 mM formic acid and methanol (90:10, v/v). Unless otherwise mentioned the flow rate used was 0.8 mL/min.

2.10. Kinetic parameters and statistical analyses

For high SMX content (5 and 10 mM) specific growth rates (μ) were calculated based on the following Eq. (1):

$$X = X_0 e^{\mu t}, \quad (1)$$

where t represents time, X biomass and X_0 biomass at $t = 0$. The biomass yield (Y) was determined through the Eq. (2):

$$Y = \frac{X - X_0}{S_0 - S}, \quad (2)$$

where S and S_0 represent the substrate content at time t and $t = 0$, respectively. Specific SMX degradation rates (r_s) were estimated as follows using Eq. (3):

$$r_s = \frac{\mu}{Y}. \quad (3)$$

For low SMX content (0.6 mM) specific degradation rates were obtained by simple linear regression of the data using SPSS (SPSS IBM, New York, U.S.A). One-way ANOVA and Student's t -test (both with $\alpha = 0.05$) using R (R Development Core Team, 2013) were used to compare the kinetic parameters data.

3. Results

3.1. Enrichment of a SMX-degrading microbial culture

Two cultures enriched from activated sludge and treated domestic wastewater demonstrated ability to degrade SMX. Because both cultures originated from samples collected from the same place and date and had identical SMX-degrading capacity, they were pooled resulting in the mixed culture 78. Since the isolation of SMX degraders from culture 78 was not possible, a further enrichment was necessary leading to culture C78 (Fig. 1). This culture, produced by serial dilutions was also able to degrade SMX with production of CO₂ (Fig. 2). After 14 days of incubation, of the 60% radioactivity recovered, ~68% was in the CO₂ trap, ~17% in the biomass, and ~15% in the cell-free culture supernatant (Fig. 2). The HPLC analysis of the culture supernatant revealed that 3A5MI was produced in stoichiometric proportion to the degraded sulfonamide (data not shown).

3.2. Composition of the enrichment cultures and of the defined mixed culture C78S

The composition of the SMX-degrading cultures over the enrichment procedure was followed by DGGE band patterning (Fig. 3). Culture 78 showed the most complex DGGE pattern with 15 bands, while its subculture C78 contained 9 bands (Fig. 3). However, further enrichment was necessary to isolate SMX degraders (Fig. 1). The combination of cultivation, phenotypic characterization, and DGGE analyses revealed that culture C78S comprised four groups of isolates, whose representatives were herein named PR1-4. The bands corresponding to strains PR1 (bands a-d), PR2 (band e), and PR3 (b and f) can be observed in the DGGE profile of culture C78S (Fig. 3). The faintness of the bands corresponding to strains PR1- 4 in the DGGE patterns of cultures 78 and C78 indicate that these

organisms were at low abundance in the mixed cultures before the final enrichment procedure.

The isolates of culture C78S were identified as *Achromobacter denitrificans* PR1 (99.9% 16S rRNA gene sequence similarity with *Achromobacter denitrificans* DSM 30026^T), *Ochrobactrum intermedium* PR2 (99.7% with *Ochrobactrum intermedium* LMG 3301^T), *Pseudoxanthomonas indica* PR3 (99.0% with *Pseudoxanthomonas indica* P15^T), and *Agromyces soli* PR4 (99.9% with *Agromyces soli* MJ21^T).

3.3. Degradation of SMX by mixed culture C78S

Aerobic degradation of SMX by mixed culture C78S was followed for 14 days in MMBN with 0.6 mM of SMX as the single source of carbon and energy (Fig. 4a). SMX was degraded with the stoichiometric accumulation of 3A5MI and no other metabolites were detected.

Despite the low biomass yields, suggesting that SMX was not a readily utilized carbon source, culture C78S was able to remove 5 and 10 mM SMX to values of about 0.03 and 0.1 mM after 103 and 188 days of incubation, respectively (Table 1). However, at these high SMX concentrations the specific degradation rate was 70% lower than with 0.6 mM SMX. The hypothesis that 3A5MI accumulated during SMX degradation could inhibit culture C78S was tested. In the presence of 20 mM 3A5MI, two-times the concentration accumulated after the degradation of 10 mM SMX, the specific rate of 0.6 mM SMX degradation was 28% lower than when that metabolite was not added (Table 1). Together, these results suggest that at very high concentrations SMX degradation may be delayed, in part due to a slight retro-inhibition by the product.

In the presence of succinate, SMX degradation showed a diauxic pattern, beginning only after the stationary phase (Fig. 5a). This suggests antibiotic degradation inhibition when other carbon sources are present. However, succinate supplementation, probably due to the existence of higher biomass yields, led to a faster SMX depletion (28.5 h) than when the antibiotic was the single carbon source. Moreover, in the absence of succinate, trace amounts of SMX (3.9 1.9 μ M) were detected even after 14 days of incubation (Fig. 4a). In the presence of succinate, SMX specific degradation rates were lower than when SMX was the single source of carbon and energy (64.4 ± 1.9 and 127.2 ± 4.0 μ molSMX/gcell dry weight h, respectively). Such differences may be explained by the higher biomass content (~ 10 x) obtained in the presence of succinate than when SMX was the single source of carbon and energy available (457 ± 3 and 46 ± 1 mg/L, respectively). Culture C78S could not grow or degrade SMX under the anaerobic conditions tested.

3.4. Degradation of SMX and other sulfonamides by bacterial isolates

In pure culture, only strain PR1 was able to degrade SMX as a sole source of carbon and energy (Fig. 4b) and also of nitrogen (data not shown). As observed for mixed culture C78S, SMX degradation by strain PR1 led to the accumulation of 3A5MI. Strain PR1 presented a lower specific degradation rate than culture C78S (73.1 ± 8.7 and $127.2 \pm 4.0 \mu\text{molSMX/gcell dry weight h}$, respectively), suggesting that the mixed culture might supply some co-factors. Indeed, the addition of a mixture of amino acids in trace amounts (Fig. 4c) significantly enhanced the degradation of SMX by strain PR1 ($188.9 \pm 7.4 \mu\text{molSMX/gcell dry weight h}$) and the fastest removal of SMX (56 h) to values close to the detection limit (Fig. 4d) and highest specific degradation rate were observed when amino acids, vitamins, and nitrogen bases were added ($237.2 \pm 18.5 \mu\text{molSMX/gcell dry weight h}$). These values are close to those observed for defined culture C78S, suggesting that the addition of micronutrients or of strains PR2, PR3, and PR4 may have a similar effect on the kinetics of SMX degradation by culture PR1. However, although micronutrients may have contributed to accelerate the degradation, apparently they did not contribute to cell growth, since the observed high specific degradation rate was explained by the low biomass yield (maximal biomass content of 80 mg/L). These results suggest that strains PR2, PR3, and PR4, although not active on SMX degradation, may enhance strain PR1 degrading activity by providing beneficial co-factors.

Similarly to what was observed for the mixed culture, also for strain PR1 the presence of 5 mM succinate led to a delay in the beginning of SMX consumption (Fig. 5b). Again, the specific degradation rate was significantly lower in the presence of succinate than in its absence (54.8 ± 3.7 and $73.1 \pm 8.7 \mu\text{molSMX/gcell dry weight h}$, respectively).

A possible NADH-dependent hydroxylation step on SMX degradation, as was described for *Microbacterium* sp. BR1 [28], was tested in crude cell-free extracts of strain PR1. In opposition to resting cells, which could remove SMX up to the detection limit, cell-free crude extracts of strain PR1 with 3 times higher protein concentration did not show significant SMX-degrading activity. When compared with abiotic controls, cell-free extracts of strain PR1 did not show ability to degrade SMX neither in the absence nor in the presence of NADH at concentrations up to 4 mM.

Strain PR1 was able to degrade all the anti-microbial sulfonamides tested. Under the conditions tested, the initial concentrations of 0.1 mM of sulfapyridine, sulfamethazine, sulfamethoxypyridazine or sulfadiazine were removed to levels below the limit of detection (0.1 μM) after 56 h of incubation. For sulfathiazole and sulfadimethoxine about half of the initial concentration was still detected after the incubation period. By contrast, sulfacetamide and the hypoglycaemic and diuretic agents tested were not degraded (i.e., less than 12%,

considering abiotic losses) (Table 2).

In spite of the few descriptions of bacteria able to degrade SMX, this is the second study demonstrating the ability of a member of the genus *Achromobacter* to transform this drug [21]. In order to test if this is a characteristic of the genus, several strains isolated from other sources were also assayed. Although differing in terms of tolerance to 0.6 mM SMX, apart from strain PR1, none of the tested strains degraded the antibiotic (Table 3).

4. Discussion

The enrichment culture 78 showed a high richness of bacteria able to tolerate 320 mg/L SMX (Fig. 3) [the minimum inhibitory concentration is estimated to be as high as 512 mg/L, [29]]. However, most of the members of culture 78, enriched from the activated sludge and/or treated wastewater samples, were not able to degrade this antibiotic. In spite of the low abundance, *Achromobacter denitrificans* PR1 was stable in the SMX tolerant microbial community, being the only organism observed to mineralize the aniline moiety of SMX with the accumulation of 3A5MI.

Biodegradation of sulfonamide antibiotics, including SMX, by activated sludge has been reported [14–18,30]. Indeed, several bacterial strains capable of degrading sulfonamide antibiotics were isolated from activated sludge [21–23,30]. However, the extent of removal of SMX during wastewater treatment varies considerably (0–100%) [2]. Such variation may be attributed to conditions affecting physical processes, such as adsorption onto suspended solids, including biomass, and also to differences in the bacterial community composition, in particular the density of SMX degraders among different wastewater treatment plants [2,31].

The selection of a consortium degrading SMX led to the isolation of four bacterial strains, affiliated to different *Proteobacteria* classes (*Beta*-, *Alpha*-, *Gamma*-*proteobacteria*, PR1-PR3) and the phylum *Actinobacteria* (PR4). Although only strain PR1 had ability to degrade SMX, the other co-isolates had probably the role of supplying some relevant co-factors, explaining the stability of this four-member consortium and the improvement of the biodegradation process. Previous studies have reported *Achromobacter* spp. as able to degrade SMX [21] and sulfamethazine [30]. *Achromobacter* sp. strain BR3 was recovered from a lab-scale membrane bioreactor acclimatized to several pharmaceuticals, including SMX [21]. Similarly to strain PR1, strain BR3 is able to mineralize approximately 44% of the initial 0.5 mM aniline ring-¹⁴C-labeled SMX after 16 days of incubation, using the antibiotic as the single source of carbon and energy [21]. However, the kinetic parameters of SMX degradation by strain BR3 were not reported. Furthermore, it is unknown whether strain BR3 shares with strain PR1 the

capacity to tolerate concentrations of SMX as high as 10 mM, while maintaining the biodegradative ability, and if SMX is degraded with the concomitant accumulation of 3A5MI. Indeed, inhibition of degradation when the initial SMX concentration was higher than 0.55 mM was reported for *P. psychrophila* HA-4 [23]. The fact that independent studies led to the isolation of *Achromobacter* strains able to degrade sulfonamides suggested that this property could be a characteristic of the genus. However, this is not the case and SMX degradation may be an adaptive mechanism [21,22].

SMX degradation is also observed in other *Proteobacteria*, such as *Pseudomonas* spp., *Brevundimonas* sp., *Variovorax* sp., and *Ralstonia* sp., and *Actinobacteria*, such as *Microbacterium* sp. and *Rhodococcus* sp. [21–23]. Also a versatile peroxidase from the ligninolytic fungus *Bjerkandera adusta* showed SMX breakdown activity [32]. The accumulation of 3A5MI, resultant of aerobic SMX degradation, seems to be common, being observed also in *Microbacterium* sp. BR1 [28], activated sludge [18] or produced by the above mentioned peroxidase [32]. Nevertheless, different reaction mechanisms may be involved on SMX breakdown with accumulation of this metabolite. Indeed, the co-accumulation of 3A5MI, aniline, 4-aminothiophenol, and sulfanilamide during SMX degradation by *Pseudomonas psychrophila* HA-4 suggests the occurrence of different degradation pathways [23].

SMX biodegradation may occur in the presence or absence of additional carbon sources either in activated sludge [14,18] or axenic cultures [22]. However, it has been suggested that additional carbon sources may delay the process also in activated sludge [14,18]. These previous observations and the results of this study suggest that the sequential use of carbon sources may hamper SMX biodegradation. This effect is particularly relevant in wastewater environments characterized by high organic loads.

Because only a few recent studies have reported the biodegradation of sulfonamides, the information of the genes, enzymes, and mechanisms involved is still scarce. Up to now, a single molecular mechanism of SMX breakdown is described for the actinobacterium *Microbacterium* sp. strain BR1 [28]. In this organism, a NADH dependent type I *ipso*-hydroxylation results in the SMX fragmentation [28]. Using the same mechanism, *Microbacterium* sp. strain BR1 is able to degrade other sulfonamides, as long as the sulfonyl bonded heteroatom-containing moieties, such as 3A5MI, can act as moderate leaving groups [28]. Strain PR1 showed a similar pattern of sulfonamide breakdown, degrading all the sulfonamides with an amine hetero-aromatic moiety, but not those containing poor leaving groups such as urea (glimepiride, glibenclamide) or NH₂ of the sulfanilamide group (furosemide) (Table 2). However, unlike *Microbacterium* sp. BR1, PR1 cell-free extracts did not show significant degrading activity, irrespective of NADH presence, suggesting that different co-factors may be involved on SMX breakdown in these organisms. The hydroxylation of

substituted aromatic compounds can be promoted by different catalysts, such as flavoprotein monooxygenases [33,34] or cytochromes P450 [35]. Therefore, further studies are needed to elucidate if the catalysts involved on sulfonamide degradation by strains PR1 and BR1 belong to one of these protein families. The further assessment of the feasibility of using immobilized strain PR1 biomass in dedicated batch reactors, or of catalysts thereof, downstream of an activated sludge process is also needed. Indeed, avoidance of environmental contamination with antibiotic resistant strains is of utmost importance. Moreover, the use of catalysts instead of all organisms could be more efficient, avoiding the observed inhibitory effect produced by other organic carbon sources.

5. Conclusions

Several successive enrichment steps were required to isolate a bacterial strain able to degrade sulfonamides, suggesting the low abundance of these organisms in wastewater environments. The degrading organism was identified as *Achromobacter denitrificans* PR1, but SMX degradation was not common to *Achromobacter* spp. from other sources.

SMX was degraded with the stoichiometric accumulation of 3A5MI and was observed only when other carbon sources were depleted. Degradation seems to depend on the structure of the sulfonyl side-chain moiety, but further studies are needed elucidate the sulfonamide degradation by this organism.

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References

- [1] S.S.A. Shah, G. Rivera, M. Ashfaq, Recent advances in medicinal chemistry of sulfonamides. Rational design as anti-tumoral, anti-bacterial and anti- inflammatory agents, Mini. Rev. Med. Chem. 13 (2013) 70–86.
- [2] I. Michael, L. Rizzo, C.S. McArdell, C.M. Manaia, C. Merlin, T. Schwartz,

C. Dagot, D. Fatta-Kassinos, Urban wastewater treatment plants as hotspots for the release of antibiotics in the environment: a review, *Water Res.* 47 (2013) 957–995.

[3] A.E. Cribb, S.P. Spielberg, Sulfamethoxazole is metabolized to the hydroxy-amine in humans, *Clin. Pharmacol. Ther.* 51 (1992) 522–526.

[4] X. Peng, Z. Wang, W. Kuang, J. Tan, K. Li, A preliminary study on the occurrence and behavior of sulfonamides, ofloxacin and chloramphenicol antimicrobials in wastewaters of two sewage treatment plants in Guangzhou, China, *Sci. Total Environ.* 371 (2006) 314–322.

[5] D.W. Kolpin, E.T. Furlong, M.T. Meyer, E.M. Thurman, S.D. Zaugg, L.B. Barber, H.T. Buxton, Pharmaceuticals, hormones, and other organic wastewater con-taminants in U.S. streams, 1999–2000: a national reconnaissance, *Environ. Sci. Technol.* 36 (2002) 1202–1211.

[6] K.K. Barnes, D.W. Kolpin, E.T. Furlong, S.D. Zaugg, M.T. Meyer, L.B. Barber, A national reconnaissance of pharmaceuticals and other organic wastewater con-taminants in the United States—I) groundwater, *Sci. Total Environ.* 402 (2008) 192–200.

[7] N. Laville, S. Aït-Aïssa, E. Gomez, C. Casellas, J.M. Porcher, Effects of human pharmaceuticals on cytotoxicity, EROD activity and ROS production in fish hepatocytes, *Toxicology* 196 (2004) 41–55.

[8] M.J. García-Galán, M.S. Díaz-Cruz, D. Barceló, Occurrence of sulfonamide residues along the Ebro river basin: removal in wastewater treatment plants and environmental impact assessment, *Environ. Int.* 37 (2011) 462–473.

[9] A.G. Trovó, R.F.P. Nogueira, A. Agüera, C. Sirtori, A.R. Fernández-Alba, Photodegradation of sulfamethoxazole in various aqueous media: persistence, toxicity and photoproducts assessment, *Chemosphere* 77 (2009) 1292–1298.

[10] L. Jiang, X. Hu, T. Xu, H. Zhang, D. Sheng, D. Yin, Prevalence of antibiotic resistance genes and their relationship with antibiotics in the Huangpu River and the drinking water sources, Shanghai, China, *Sci. Total Environ.* 458–460 (2013) 267–272.

[11] P.T.P. Hoa, S. Managaki, N. Nakada, H. Takada, A. Shimizu, D.H. Anh, P.H. Viet, S. Suzuki, Antibiotic contamination and occurrence of antibiotic-resistant bacteria in aquatic environments of northern Vietnam, *Sci. Total Environ.* 409 (2011) 2894–2901.

[12] C.M. Manaia, I. Vaz-Moreira, O.C. Nunes, Antibiotic resistance in waste- and surface waters and human health implications, in: D. Barceló (Ed.), *The Handbook of Environmental Chemistry*, Springer-Verlag, Berlin Heidelberg, 2012, pp. 173–212.

[13] X.X. Zhang, T. Zhang, H. Fang, Antibiotic resistance genes in water environment, *Appl. Microbiol. Biotechnol.* 82 (2009) 397–414.

- [14] P. Drillia, S.N. Dokianakis, M.S. Fountoulakis, M. Kornaros, K. Stamatelatou, G. Lyberatos, On the occasional biodegradation of pharmaceuticals in the activated sludge process: the example of the antibiotic sulfamethoxazole, *J. Hazard. Mater.* 122 (2005) 259–265.
- [15] T.H. Yu, A.Y.C. Lin, S.K. Lateef, C.F. Lin, P.Y. Yang, Removal of antibiotics and non-steroidal anti-inflammatory drugs by extended sludge age biological process, *Chemosphere* 77 (2009) 175–181.
- [16] S.F. Yang, C.F. Lin, C.J. Wu, K.K. Ng, A. Yu-Chen Lin, P.K. Andy Hong, Fate of sulfonamide antibiotics in contact with activated sludge—sorption and biodegradation, *Water Res.* 46 (2012) 1301–1308.
- [17] N. Collado, G. Buttiglieri, E. Marti, L. Ferrando-Climent, S. Rodriguez-Mozaz, D. Barceló, J. Comas, I. Rodriguez-Roda, Effects on activated sludge bacterial community exposed to sulfamethoxazole, *Chemosphere* 93 (2013) 99–106.
- [18] E. Müller, W. Schüssler, H. Horn, H. Lemmer, Aerobic biodegradation of the sulfonamide antibiotic sulfamethoxazole by activated sludge applied as co-substrate and sole carbon and nitrogen source, *Chemosphere* 92 (2013) 969–978.
- [19] H. Gauthier, V. Yargeau, D.G. Cooper, Biodegradation of pharmaceuticals by *Rhodococcus rhodochrous* and *Aspergillus niger* by co-metabolism, *Sci. Total Environ.* 408 (2010) 1701–1706.
- [20] S. Larcher, V. Yargeau, Biodegradation of sulfamethoxazole by individual and mixed bacteria, *Appl. Microbiol. Biotechnol.* 91 (2011) 211–218.
- [21] H. Bouju, B. Ricken, T. Beffa, P.F.X. Corvini, B.A. Kolvenbach, Isolation of bacterial strains capable of mineralizing sulfamethoxazole from an acclimated membrane bioreactor, *Appl. Environ. Microbiol.* 78 (2012) 277–279.
- [22] B. Herzog, H. Lemmer, H. Horn, E. Muller, Characterization of pure cultures isolated from sulfamethoxazole-acclimated activated sludge with respect to taxonomic identification and sulfamethoxazole biodegradation potential, *BMC Microbiol.* 13 (2013) 276.
- [23] B. Jiang, A. Li, D. Cui, R. Cai, F. Ma, Y. Wang, Biodegradation and metabolic pathway of sulfamethoxazole by *Pseudomonas psychrophila* HA-4, a newly isolated cold-adapted sulfamethoxazole-degrading bacterium, *Appl. Microbiol. Biotechnol.* 98 (2014) 4671–4681.
- [24] L. Barreiros, B. Nogales, C.M. Manaia, A.C.S. Ferreira, D.H. Pieper, M.A. Reis, O.C. Nunes, A novel pathway for mineralization of the thiocarbamate herbicide molinate by a defined bacterial mixed culture, *Environ. Microbiol.* 5 (2003) 944–953.
- [25] O.C. Nunes, M.M. Donato, M.S. da Costa, Isolation and characterization of *Rhodothermus* strains from S. Miguel, Azores, *Syst. Appl. Microbiol.* 15 (1992) 92–97.

- [26] O. Maltseva, P. Oriel, Monitoring of an alkaline 2,4,6-trichlorophenol-degrading enrichment culture by DNA fingerprinting methods and isolation of the responsible organism, *haloalkaliphilic Nocardioides* sp. strain M6, Appl. Environ. Microbiol. 63 (1997) 4145–4149.
- [27] L. Barreiros, A. Fernandes, A.C.S. Ferreira, H. Pereira, M.M.S.M. Bastos, C.M. Manaia, O.C. Nunes, New insights into a bacterial metabolic and detoxifying association responsible for the mineralization of the thiocarbamate herbicide molinate, Microbiology 154 (2008) 1038–1046.
- [28] B. Ricken, P.F.X. Corvini, D. Cichocka, M. Parisi, M. Lenz, D. Wyss, P.M. Martínez-Lavanchy, J.A. Müller, P. Shahgaldian, L.G. Tulli, H.-P.E. Kohler, B.A. Kolvenbach, *ipso*-Hydroxylation and subsequent fragmentation: a novel microbial strategy to eliminate sulfonamide antibiotics, Appl. Environ. Microbiol. 79 (2013) 5550–5558.
- [29] CLSI., Performance standards for anti-microbial susceptibility testing; twenty-second informational supplement, in: CLSI document M100-S22, Clinical and Laboratory Standards Institute, Wayne, PA, 2012.
- [30] M. Huang, S. Tian, D. Chen, W. Zhang, J. Wu, L. Chen, Removal of sulfamethazine antibiotics by aerobic sludge and an isolated *Achromobacter* sp. S-3, J. Environ. Sci. 24 (2012) 1594–1599.
- [31] B. Halling-Sørensen, S. Nors Nielsen, P.F. Lanzky, F. Ingerslev, H.C. Holten Lützhøft, S.E. Jørgensen, Occurrence, fate and effects of pharmaceutical substances in the environment – a review, Chemosphere 36 (1998) 357–393.
- [32] G. Eibes, G. Debernardi, G. Feijoo, M.T. Moreira, J. Lema, Oxidation of pharmaceutically active compounds by a ligninolytic fungal peroxidase, Biodegradation 22 (2011) 539–550.
- [33] A.W. Porter, A.G. Hay, Identification of *opdA*, a gene involved in biodegradation of the endocrine disrupter octylphenol, Appl. Environ. Microbiol. 73 (2007) 7373–7379.
- [34] A.W. Porter, B.R. Campbell, B.A. Kolvenbach, P.F.X. Corvini, D. Benndorf, G. Rivera-Cancel, A.G. Hay, Identification of the flavin monooxygenase responsible for *ipso* substitution of alkyl and alkoxyphenols in *Sphingomonas* sp. TTNP3 and *Sphingobium xenophagum* Bayram, Appl. Microbiol. Biotechnol. 94 (2012) 261–272.
- [35] F.P. Guengerich, Common and uncommon cytochrome P450 reactions related to metabolism and chemical toxicity, Chem. Res. Toxicol. 14 (2001) 611–650.
- [36] I. Vaz-Moreira, C. Egas, O.C. Nunes, C.M. Manaia, Bacterial diversity from the source to the tap: a comparative study based on 16S rRNA gene-DGGE and culture-dependent methods, FEMS Microbiol. Ecol. 83 (2013) 361–374.

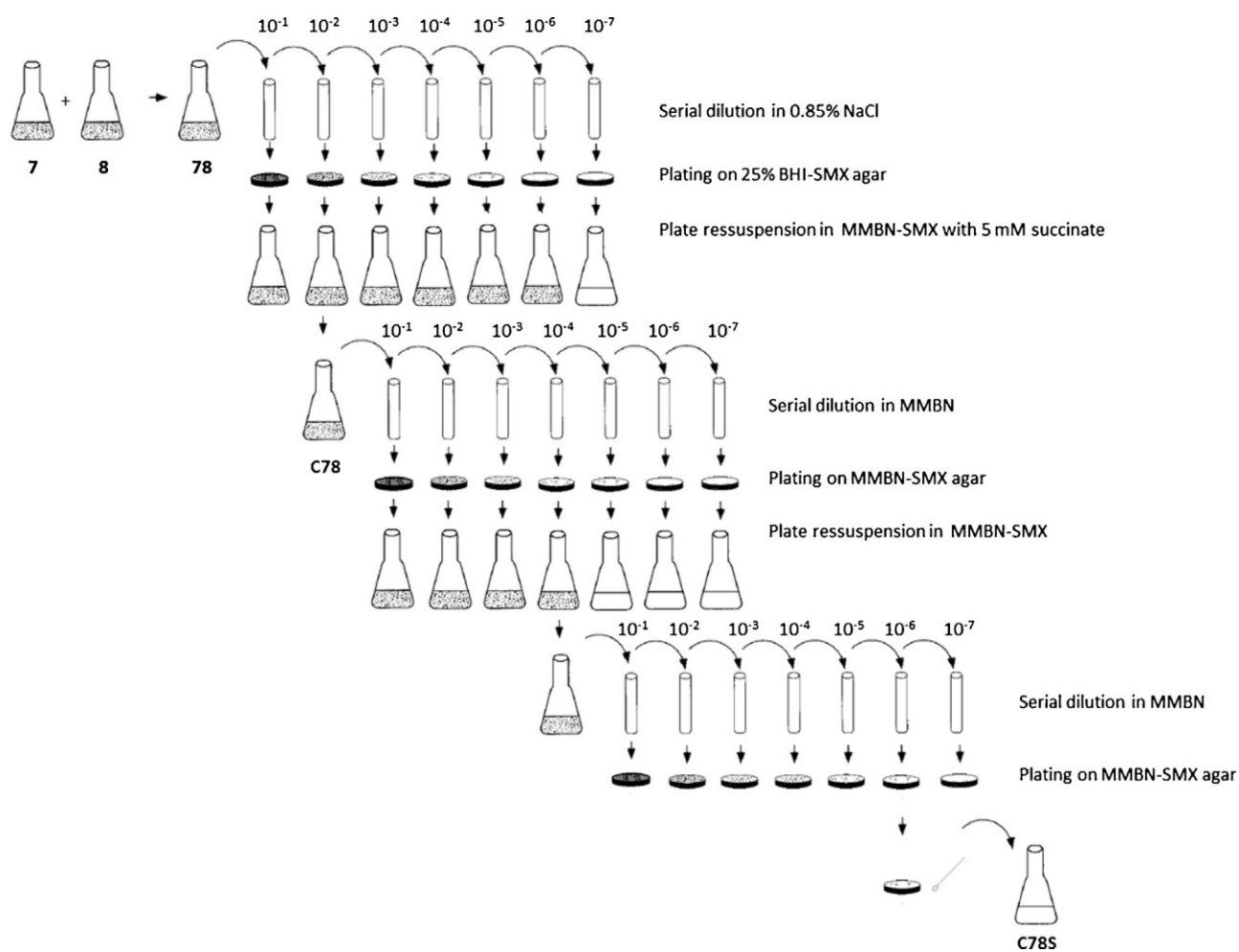


Fig. 1. Enrichment and serial dilution plate re-suspension of the SMX-degrading cultures.

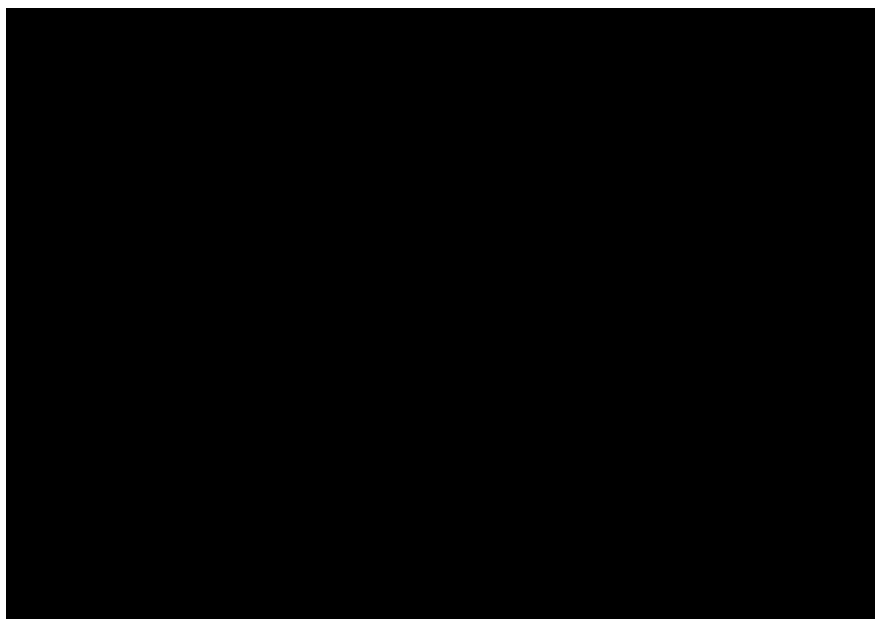


Fig. 2. Distribution of [aniline ring- ^{14}C]-labeled SMX radioactivity recovered in CO_2 traps (\otimes), supernatant (\bullet), and biomass fractions (\circ) throughout 25 days of incubation of culture C78 in MMBN with 0.6 mM SMX as sole carbon source. Radioactivity in the abiotic supernatant (\blacksquare) and CO_2 traps (\boxtimes). Bars represent standard deviation of three independent experiments.

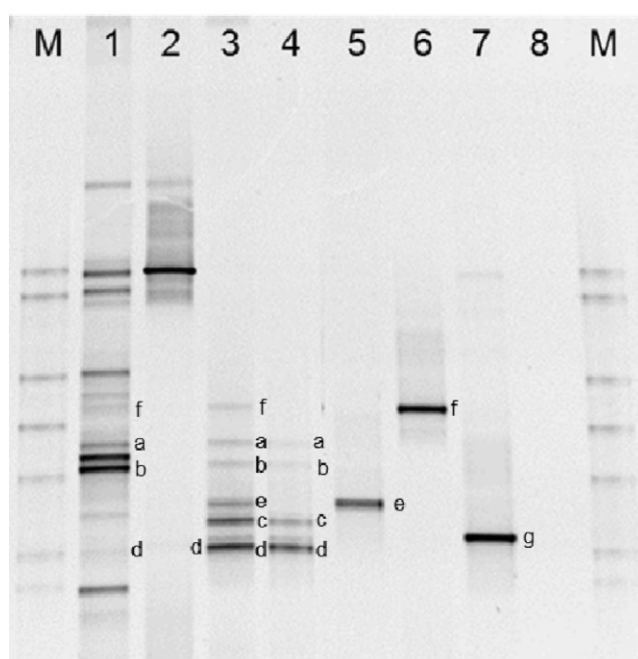


Fig. 3. 16S rRNA gene-DGGE profiles of mixed cultures 78 (lane 1), C78 (lane 2), C78S (lane 3) and strains PR1 (lane 4), PR2 (lane 5), PR3 (lane 6), and PR4 (lane 7) of culture C78S. Negative control (lane 8). M, marker.

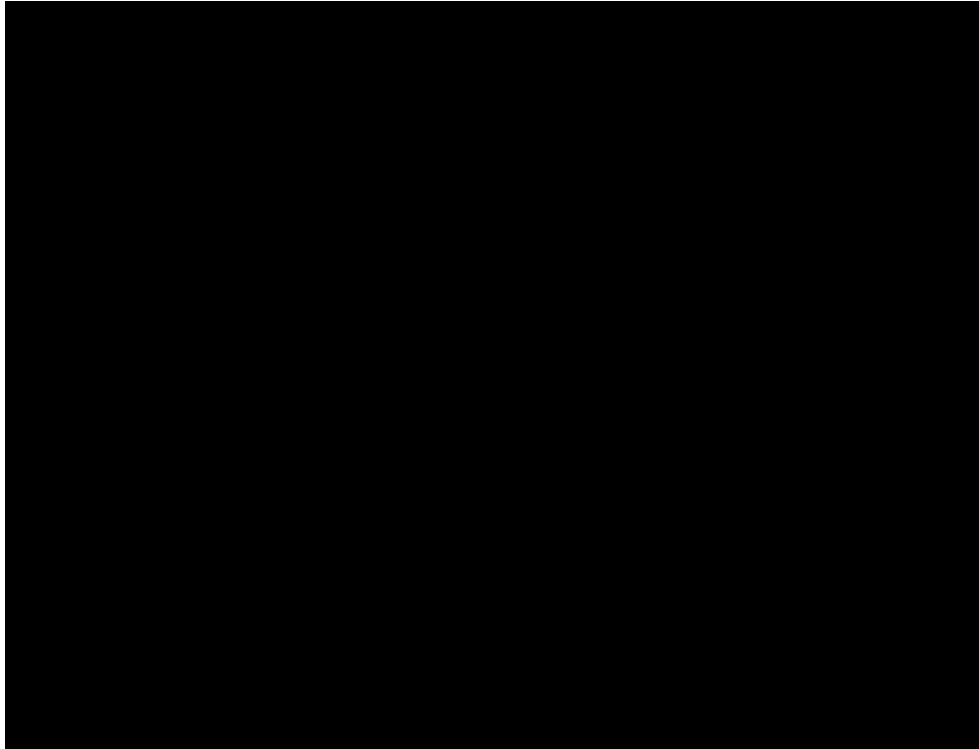


Fig. 4. Concentration of SMX (e), 3A5MI (·à), biomass content (+), and SMX abiotic losses (\pm) over 14 days of incubation in MMBN with 0.6 mM of SMX. Mixed culture C78S (a); strain PR1 without any supplement (b) and with amino acids (c) and with amino acids, vitamins and nitrogenous bases (d). Bars represent standard deviation of three independent experiments.

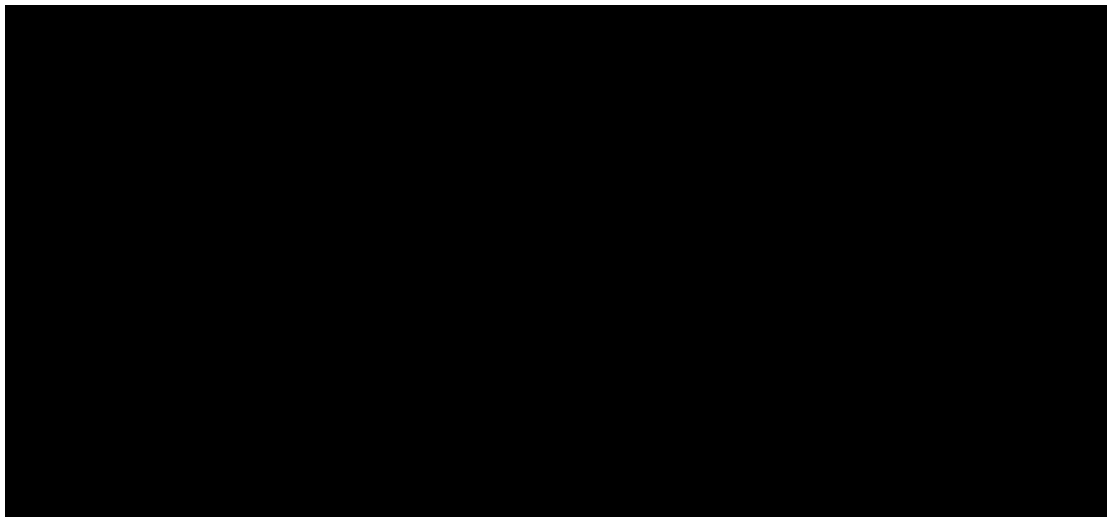


Fig. 5. Concentration of SMX (e), 3A5MI (·à), biomass content (+), and SMX abiotic losses (\pm) over 30.5 and 41.5 h of incubation in MMBN with 0.6 mM SMX and 5 mM succinate for the mixed culture C78S (a) and strain PR1 (b). Bars represent standard deviation of three independent experiments.

Table 1

Specific growth (μ) and SMX degradation (r_s) rates and biomass yield (Y) of mixed culture C78S in MMBN in the presence of 0.6, 5.0 or 10.0 mM SMX or in the presence of 0.6 mM SMX and 20 mM 3A5MI or 0.6 mM SMX and 5 mM of succinate.

Kinetic parameters	SMX (mM) as sole carbon source			SMX (0.6 mM) with	
	0.6	5.0	10.0	3A5MI (20 mM)	Succinate ¹ (5 mM)
r_s ($\mu\text{mol}_{\text{SMX}}/\text{g}_{\text{cell dry weight h}}$)	127.2 ± 4.0^d	35.6 ± 0.3^a	28.8 ± 8.7^a	106.2 ± 0.2^c	64.4 ± 1.9^b
μ ($\times 10^{-4} \text{ h}^{-1}$)	0.0 ± 0.0^a	9.4 ± 0.1^c	8.5 ± 0.4^b	0.0 ± 0.0^a	0.0 ± 0.0^a
Y ($\times 10^{-2} \text{ g}_{\text{cell dry weight}}/\text{g}_{\text{SMX}}$)	0.0 ± 0.0^a	10.4 ± 0.0^b	12.3 ± 4.3^b	0.0 ± 0.0^a	0.0 ± 0.0^a
Residual SMX (μM)(days of incubation)	6.2 ± 0.8 (7)	33.0 ± 4.5 (103)	106.3 ± 13.0 (188)	49.1 ± 0.0 (6)	17.3 ± 0.8 (1)

Values are expressed as average \pm standard deviation of three independent experiments.

¹ Specific degradation rate observed after succinate degradation, in stationary phase. ^{a,b,c,d}Significantly different ($p < 0.05$) r_s ($\mu\text{mol}_{\text{SMX}}/\text{g}_{\text{cell dry weight h}}$), μ (h^{-1}) or Y ($\text{g}_{\text{cell dry weight}}/\text{g}_{\text{SMX}}$) values.

Table 2. Percentage of degradation of selected sulfonamides by *Achromobacter* sp. strain PR1 after 56 h incubation. Values are expressed as average \pm standard deviation of three independent experiments.

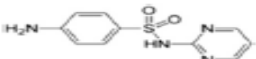
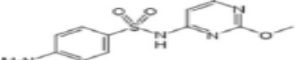
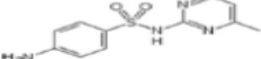

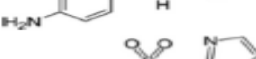


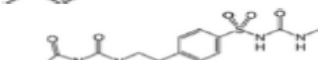
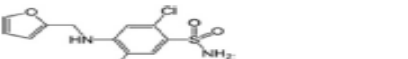
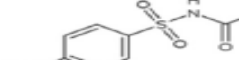

Sulfonamide	Chemical structure	Degradation (%) Strain PR1	Abiotic control
Sulfadiazine		98 \pm 2	1
Sulfadimethoxine		48 \pm 1	2
Sulfamethazine		100 \pm 0	0
Sulfamethoxypyridazine		98 \pm 2	0
Sulfapyridine		100 \pm 0	1
Sulfathiazole		47 \pm 13	1
Glibenclamide		0 \pm 0	0
Gliclazide		19 \pm 0	7
Glimepiride		0 \pm 0	0
Furosemide		1 \pm 3	0
Sulfacetamide		10 \pm 2	1

Table 3Normalized biomass (N/N_0) and percentage of SMX removal by *Achromobacter* spp. isolated from different environments

Strain	Origin	Affiliation	Normalized biomass (N/N_0)	% SMX removal
PR1	This study	<i>A. denitrificans</i>	7.8	99.1
AR32	Surface water	<i>A. denitrificans</i>	0.0	0.0
TAP _n	Household tap water	<i>A. denitrificans</i>	8.1–0.2 ^a	1.2–0.0 ^a
T11A.R6	Household tap water	<i>A. ruhlandii</i>	7.5	2.7
ON5	Enrichment culture ^b	<i>A. xylosoxidans</i> subs. <i>denitrificans</i>	9.0	0.5
Abiotic control	NA	NA	0.0	1.3

^a Range of values for 13 strains isolated from tap water [36].^b Molinate degrading mixed culture DC [24].

NA, not applicable.