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Saccharin as a sole source of carbon and energy for *Sphingomonas xenophaga* SKN

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Abstract A bacterium, strain SKN, that was able to utilize saccharin as the sole source of carbon and energy for aerobic growth, was enriched and isolated from communal sewage. The isolate was identified as a strain of *Sphingomonas xenophaga*. Saccharin was quantitatively converted to cell material, sulfate, ammonium and, presumably, CO_2. The specific rate of saccharin-dependent oxygen uptake during growth reached a maximum before the culture entered the stationary phase and then fell to undetectable levels. Saccharin was degraded only in the presence of molecular oxygen. Catechol was detected as an intermediate during degradation of saccharin in whole cells and catechol 1,2-dioxygenase was expressed inducibly during growth with saccharin. There was an apparent requirement of 2 mol O_2/mol saccharin to remove the substituents on the ring and to cleave the ring. We presume that *S. xenophaga* SKN synthesizes a multi-component saccharin dioxygenase that simultaneously cleaves off both vicinal substituents from the aromatic ring to yield catechol and the undefined precursor of CO_2 as well as sulfate and ammonium ions.

Keywords Saccharin degradation · Saccharin dioxygenase · *Sphingomonas xenophaga* · Catechol 1,2 dioxygenase

Introduction

Saccharin (2-sulfobenzoic-acid imide; Fig. 1) has been used world-wide as a food additive for over a century (von Rymon Lipinski 1995), despite the fact that its use in the USA was banned for decades, because it was classified, for non-scientific reasons, as a carcinogen (Weisburger 1996). The unaltered compound is subject to rapid, quantitative clearance in urine (von Rymon Lipinski 1995). Saccharin is also known as a product of metabolism of sulfonylurea herbicides in soil (Anderson and Dilka 1985; Environmental Protection Agency 1989), and the compound is described as persistent (European Community Directorate 2000), despite the fact that degradation in soil was demonstrated (Anderson and Dilka 1985). Moreover, many enrichment cultures able to assimilate sulfur quantitatively from saccharin have been obtained (Cook, unpublished data; see Kertesz 2000); for example, *Gordonia* sp. converts saccharin to salicylamide under these conditions (Rein and Cook 1999). Thus, the term "persistent" is misleading.

Here we report the ready utilization of saccharin, via catechol, as a source of carbon and energy by *Sphingomonas xenophaga* SKN, which was enriched and isolated from a communal sewage-treatment plant. We believe this to be the first report of the quantitative dissimilation of saccharin.

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Materials and methods

Growth media and growth conditions

A mineral-salts medium buffered with 50 mM potassium phosphate, pH 7.2, was used (Thurnheer et al. 1986). Saccharin could be autoclaved in solution and was routinely present in the growth medium at 6 mM. Enrichment cultures (3 ml in 30-ml screw cap tubes) were incubated at 30 °C on a rotary shaker in the dark; the inoculum (50 µl) was an untreated sample from the aeration tank of a sewage works. The positive enrichment culture was transferred several times into fresh selective medium, plated on selective medium solidified with agar (1.5%), and a colony was transferred to selective mineral medium. The outgrown culture was streaked on LB-agar (Gerhardt et al. 1994) and picked to selective medium. The pure culture was termed strain SKN, and was identified by standard methods under contract at the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany).

Growth experiments were done with 250-ml cultures in 1-l Erlenmeyer flasks on a rotary shaker at 30 °C. Strain SKN was grown in 4 mM saccharin/minimal-salts medium and samples were taken at intervals to determine concentrations of substrate and products,

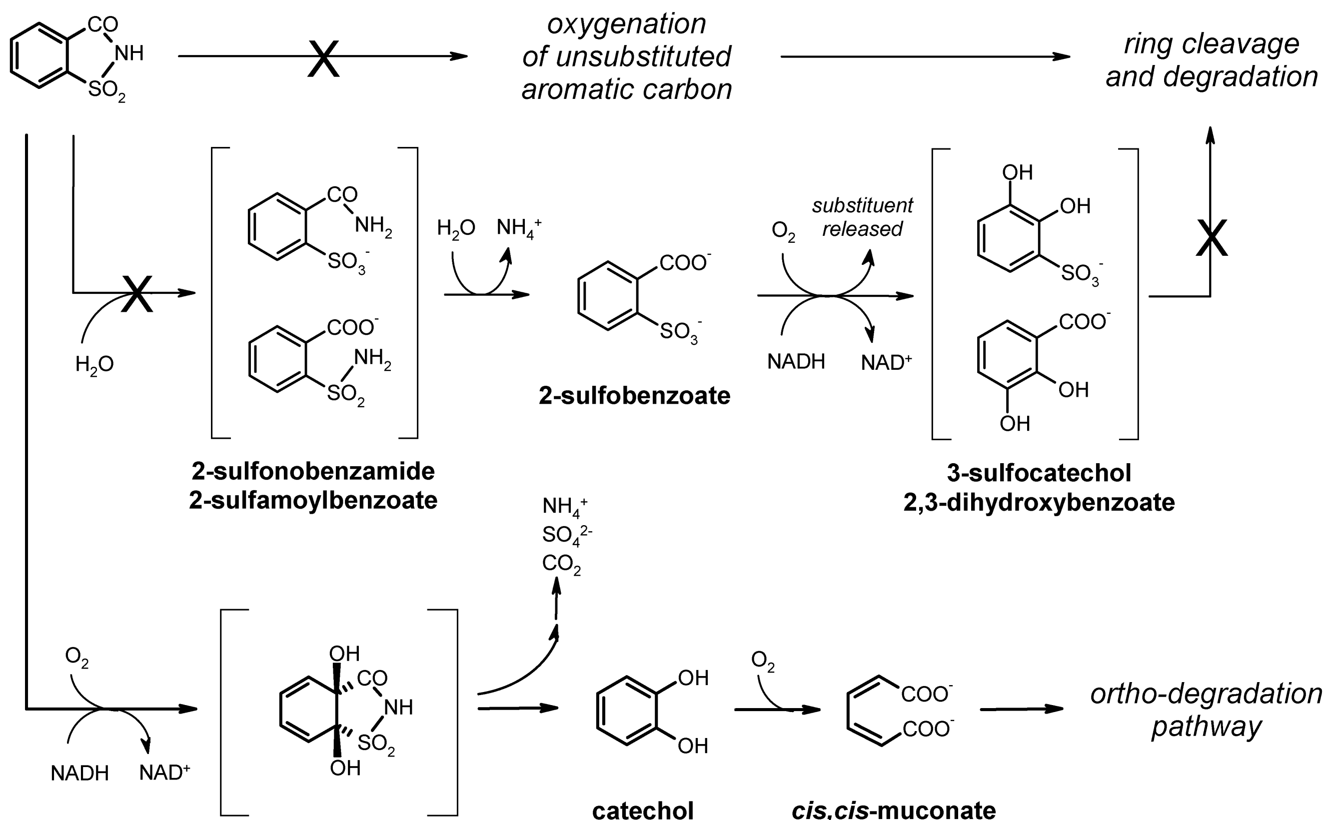

Fig. 1 Hypothetical saccharin-degrading reactions, and the observed degradative pathway via *ortho* cleavage of catechol

to measure turbidity and assay protein concentration, and to determine the specific oxygen uptake rates of the culture.

Cells from which crude extract was to be prepared were grown as in the growth experiments but were harvested (10,000×*g*, 10 min, 4 °C) at 65 mg protein ml^{-1} (OD_{580}=0.28), washed twice in chilled 50 mM potassium phosphate buffer, pH 7.2, and used immediately or stored frozen at –20 °C. Washed cells were resuspended in 50 mM Tris-HCl, pH 7.5, which contained 10% (v/v) glycerol, and disrupted by three passages through a chilled French pressure cell (138 MPa). Whole cells and debris were removed by centrifugation (16,000×*g*, 15 min, 4 °C) and the membrane fraction was removed by ultracentrifugation (100,000×*g*, 40 min, 4 °C).

Enzyme assays

Oxygen uptake was assayed in membrane-free, crude extracts in 50 mM Tris-HCl buffer, pH 7.5, at 30 °C in a Clarke-type electrode. Samples (10 µl) were periodically taken to follow substrate turnover by HPLC. Saccharin or 2-sulfobenzoate was present at 1 mM and the protein concentration was 6 mg ml^{-1}. The net reaction was not altered by the presence of 1 mM NADH. Ring-cleavage reactions in crude extract (1.2 mg protein ml^{-1}, 2 mM $FeCl_2$) were measured in the oxygen electrode after addition of 1 mM catechol, 1 mM 3-sulfocatechol, or 2,3-dihydroxybenzoate. Catechol 1,2-dioxygenase [EC 1.13.11.1.] was assayed photometrically as disappearance of catechol (Hegeman 1966). *cis,cis*-Muconate lactonizing enzyme [EC 5.5.1.1] was measured photometrically as substrate disappearance (Ornston 1966).

Analytical methods

Growth was followed as turbidity (OD_{580}=1.0=360 mg protein l^{-1}) and quantified as protein in a Lowry-type reaction (Kennedy and Fewson 1968). Protein in crude extracts was assayed by protein-dye binding (Bradford 1976). Saccharin and catechol were separated and quantified by reversed-phase HPLC (Schleheck et al. 2000): potassium phosphate buffer (10 mM, pH 2.2) was pumped at 0.5 ml min^{-1} for 1 min when a linear gradient to 60 % MeOH over 8 min was applied and maintained for 4 min. Saccharin and catechol eluted at 9.8 min and 9.3 min, respectively; 3-sulfocatechol eluted at 4.1 min, 2-sulfobenzoate at 4.8 min, 2,3-dihydroxybenzoate at 11.8 min, 4-nitrocatechol at 12.3 min, and 3-chlorocatechol at 12.8 min. Sulfate was determined turbidimetrically as a suspension of $BaSO_4$ (Sörbo 1987). Ammonium ion was determined by the Berthelot reaction (Gesellschaft Deutscher Chemiker 1996).

Cells for the assay of substrate-dependent oxygen uptake were collected by rapid centrifugation, washed, resuspended in 50 mM potassium phosphate buffer, pH 7.2, and equilibrated at 30 °C in a Clarke-type oxygen electrode; substrate was added to 1 mM and the net oxygen uptake rate was measured (see Junker et al. 1994b).

Inhibition of oxygenase activities in whole cells was examined in the oxygen electrode. 3-Chlorocatechol, 4-nitrocatechol, cetyltrimethylammonium bromide (CTAB) (see Brune and Schink 1990) and SDS were used at concentrations given in the Results. Cells were either pretreated with the inhibitor (10 min), or the inhibitor was added after the reactions had been started by addition of substrate (saccharin, catechol).

Excretion of catechol from whole cells in the presence of saccharin and 3-chlorocatechol was followed by HPLC. The reaction mixture (5 ml, 2.8 mg protein ml^{-1}) was aerated by magnetic stirring at 30 °C, and a reaction was started by the addition of saccharin (to give 0.5 mM): if appropriate, 3-chlorocatechol (to give 0.1 mM) was added after 1 min. Samples (0.1 ml) were taken at intervals and added to 0.9 ml 0.05 M phosphoric acid, and then centrifuged (10,000×*g*, 10 min). The supernatant was analyzed by HPLC.

Atmospheric oxygen was removed from 5-ml cell suspensions in 10-ml septum vials by degassing under vacuum and purging with nitrogen gas through needles in the septum. The reaction was started by addition of saccharin. Samples were taken by syringe at intervals through the septum and analyzed by HPLC.

Chemicals

Saccharin (>99 %) was from Aldrich. 3-Sulfocatechol was generated by Junker et al. (1994a). *cis,cis*-Muconate was kindly provided by the late D.W. Ribbons.

Results

Enrichment, isolation and identification of strain SKN

Minimal-salts medium with 6 mM saccharin as the sole source of carbon and energy for aerobic growth was inoculated with activated sludge from the treatment plant for communal sewage in Konstanz, Germany. The culture developed the same turbidity as the positive control (6 mM glucose) within a week; the negative control (no carbon source) showed negligible growth. The enrichment culture was passaged three times in fresh selective medium. Spent medium contained no saccharin, which was stable in an uninoculated control. The culture was yellow, which, after centrifugation, was seen to be caused by a cell-bound pigment. The majority of the mixed culture was a rod-shaped bacterium (2.0–4.0 µm in length and 0.6–0.8 µm in diameter).

The culture was plated on LB-agar and yielded large white colonies overnight; these coryneform organisms did not grow in selective medium. The enrichment culture was then plated on selective medium and large numbers of yellow colonies of rod-shaped organisms were observed within a week; white pinpoint colonies were also present. A representative yellow colony was picked to selective medium and the outgrown culture was plated on LB-medium. Yellow colonies were obtained in 4 days. The culture was considered pure after three cycles of growth in selective medium and on non-selective plates yielded homogeneous colony morphology. The isolate was termed strain SKN.

Strain SKN is a gram-negative, non-motile rod and is catalase- and oxidase-positive. A partial sequence of the 16S-rRNA gene (400 bp) of strain SKN gave 100% sequence identity with data from *Sphingomonas xenophaga*^{T} (see Stolz et al. 2000). This preliminary identification was supported by the pattern of utilization of carbon sources and confirmed by the profile of cellular fatty acids, which was identical with that of the type strain (see Stolz et al. 2000). *S. xenophaga* SKN was deposited in the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany) as DSM 14677.

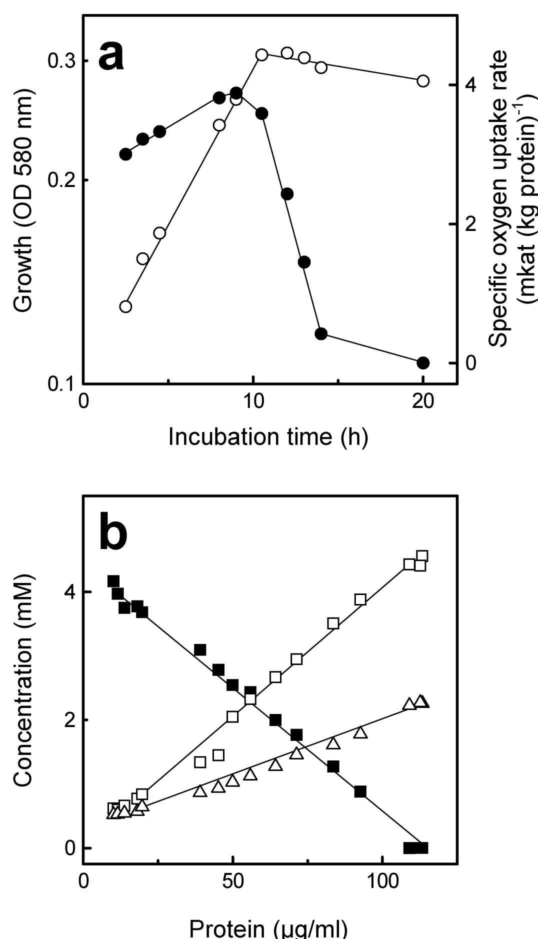

Fig. 2a, b Growth (○) of *Sphingomonas xenophaga* SKN in 4 mM saccharin/minimal-salts medium. **a** During growth, the specific oxygen uptake of whole cells (●) varied widely. **b** A linear plot shows the concentrations of saccharin (■), sulfate (□), and ammonium ion (Δ) during growth

Growth physiology

Strain SKN grew with saccharin, glucose, glycerol and succinate (present at 6–12 mM) as sole sources of carbon and energy. The organism did not utilize benzoate, salicylate, 2,3-dihydroxybenzoate, gentisate, protocatechuate, benzenesulfonate, *o*-, *m*- and *p*-sulfobenzoate, *o*-aminobenzenesulfonate, *o*-nitrobenzenesulfonate, *p*-toluenesulfonate, *p*-sulfophenol, naphthalene-2-sulfonate (each at 6 mM), or catechol (at 1 mM).

Stolz et al. (2000) reported that *S. xenophaga*^{T} rapidly lost ability to form colonies after reaching the stationary phase. We observed long lag-phases in saccharin/minimal-salts medium when the organism was transferred after entering the stationary phase, but they could be circumvented by subculturing in the exponential growth phase, or by supplementing saccharin/minimal-salts medium with 0.1 mM glucose.

S. xenophaga SKN grew in 4 mM saccharin/minimal-salts medium with a specific growth rate (μ) of 0.14 h^{-1} (Fig. 2a). Substrate utilization was concomitant with growth, as were the release of sulfate and ammonium ion (Fig. 2b). The molar growth yield was 4 g protein (mol C)$^{-1}$, which is within the range indicating quantitative substrate utilization (Cook 1987). The recovery of sulfonate sulfur

as sulfate was 95%, which corresponds to the high molar growth yield with sulfur (about 4 kg protein (mol S)$^{-1}$; Cook 1987); no sulfite was detected. The recovery of organic nitrogen as ammonium ion was 42 %, which corresponds to a molar growth yield of about 50 g protein (mol N)$^{-1}$, a normal value (Cook 1987). There is thus a complete mass balance for the utilization of saccharin. The specific degradation rate of saccharin during growth was calculated as 1.1 mkat (kg protein)$^{-1}$.

Enzymes detected and the degradative pathway

Saccharin-dependent uptake of oxygen was detected in whole cells of strain SKN during growth (Fig. 2a) and HPLC analysis of samples taken from reaction mixtures showed that saccharin was utilized during these reactions; the reaction mixture remained colorless. The specific activity rose steadily to about 3.8 mkat (kg protein)$^{-1}$ shortly before the end of growth, after which the activity fell to undetectable levels within about 6 h (Fig. 2a); this behavior is typical of many multi-component oxygenases (e.g. Junker et al. 1994a). Succinate- and glucose-grown cells showed no saccharin-dependent uptake of oxygen; thus, the putative multi-component oxygenase involved in the degradation of saccharin is presumably inducible.

Whole cells that degraded saccharin showed no oxygen uptake with 2-sulfobenzoate, 3-sulfocatechol, or 2,3-dihydroxybenzoate. In contrast, catechol caused oxygen uptake at about 8.8 mkat (kg protein)$^{-1}$; the reaction mixtures remained colorless and catechol was degraded (as measured by HPLC). This was interpreted as preliminary evidence for *ortho* ring cleavage. Glucose-grown cells showed negligible catechol-dependent oxygen uptake (0.3 mkat (kg protein)$^{-1}$), indicating that the degradation of catechol is presumably inducible: succinate-grown cells expressed a significant level of catechol 1,2-dioxygenase (3.4 mkat (kg protein)$^{-1}$).

Freshly prepared crude extract [up to 6 mg protein (ml reaction mixture)$^{-1}$] of optimally harvested, saccharin-grown cells in the presence of NADH showed neither oxygen uptake nor substrate disappearance when saccharin or 2-sulfobenzoate was tested. The additional presence of FAD or FMN had negligible effect. Catechol caused oxygen uptake with a specific activity of about 3.3 mkat (kg protein)$^{-1}$, whereas 3-sulfocatechol or 2,3-dihydroxybenzoate did not. Catechol disappeared (as followed by HPLC) and the reaction mixture remained colorless.

The *ortho* cleavage of catechol was confirmed photometrically. Formation of putative *cis*,*cis*-muconate (measured as A_{257}) was concomitant with the disappearance of catechol (A_{220}). The activity of a muconate lactonizing enzyme was detected and quantified at about 1.1 mkat (kg protein)$^{-1}$.

Crude extract had an oxygen uptake of 1.1 mol O_2 (mol catechol)$^{-1}$. The uptake by whole cells was higher, 1.4 mol O_2 (mol catechol)$^{-1}$, suggesting that some intermediates were completely oxidized in the cell suspension. The oxygen uptake of whole cells with saccharin was 2.9 mol O_2 (mol saccharin)$^{-1}$. This result could be explained by the presence in whole cell of two active dioxygenases, a multi-component, ring-activating enzyme, which yielded catechol, and catechol 1,2-dioxygenase. We presume that the additional oxygen was involved in respiration.

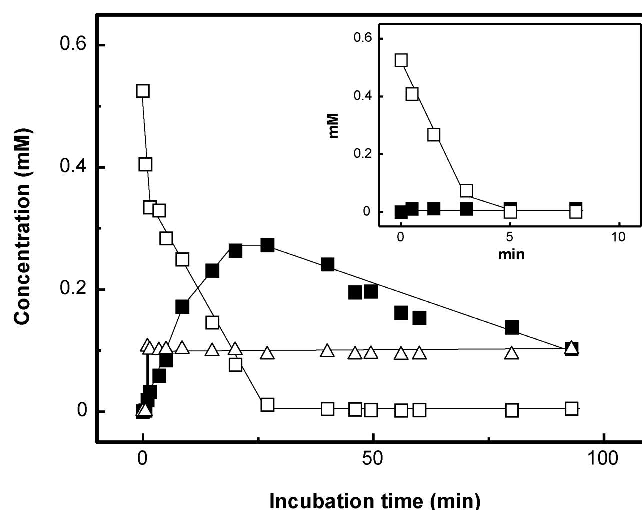

Fig. 3 Degradation of saccharin by suspensions of resting cells in the presence and absence (*inset*) of 3-chlorocatechol. The reaction was started by the addition of saccharin, and 3-chlorocatechol was added after 1 min. ❑ Saccharin, ■ catechol, and Δ 3-chlorocatechol

Whole cells incubated under anoxic conditions (N_2) did not metabolize saccharin, and no intermediate accumulated (as determined by HPLC). On addition of air, saccharin was degraded (not shown). This indicated that the first metabolic reaction involving saccharin is dependent on molecular oxygen, and we postulate a saccharin dioxygenase.

Saccharin was degraded by whole cells without any visible excretion products (Fig. 3, inset). Saccharin and catechol dioxygenases were inactive in whole cells pretreated with 4-nitrocatechol or 3-chlorocatechol [35 µmol (mg protein)$^{-1}$]. When 3-chlorocatechol (or 4-nitrocatechol, not shown) was added after the reactions had been started, saccharin dioxygenase was subject to >90 % inhibition [from 0.9 mkat (kg protein)$^{-1}$ to 80 µkat (kg protein)$^{-1}$] and release of a transient intermediate was detected (Fig. 3). This intermediate was identified as catechol since it co-eluted from the HPLC with authentic catechol and had the same UV-spectrum as authentic material. Catechol was degraded at a specific rate of 22 µkat (kg protein)$^{-1}$, measured after exhaustion of the saccharin (at 26 min; Fig. 3); catechol 1,2-dioxygenase was thus subject to about 99 % inhibition. Extrapolation of the catechol degradation curve to the time of addition of the inhibitor indicated unit stoichiometry for the formation of catechol from saccharin.

Whole cells were permeabilized with CTAB [0.2 mg CTAB (mg protein)$^{-1}$] (or SDS), and this condition was detected as loss of endogenous respiration in the oxygen electrode. Whereas catechol 1,2-dioxygenase was active,

saccharin or 2-sulfobenzoate added to permeabilized cells caused no oxygen uptake, and the presence of NADH, or the additional presence of FMN, FAD and Fe^{2+}, had no effect. Saccharin dioxygenase was inactivated immediately when CTAB was added to whole cells.

Multi-component oxygenases are sometimes expressed at very high levels and can be detected by SDS-PAGE in crude extracts (e.g. Junker et al. 1996). We detected two proteins (approximately 75 kDa and 98 kDa, not shown) that were specifically induced in saccharin-grown cells, but their function was not determined.

Discussion

The results presented here show that saccharin is subject to complete dissimilation (Fig. 2a, b). The compound can also be utilized as a source of sulfur by many bacteria (Rein and Cook 1999; Cook, unpublished data), thus the term "persistent" (see European Commisison Directorate E1 2000) is not generally valid. Several bacteria able to assimilate sulfur from saccharin have been isolated from soil (Cook, unpublished data), suggesting that saccharin will tend to be attacked in soils without sulfate fertilization (see Kertesz 2000). It is also likely that saccharin is degraded in the aeration tank of sewage treatment plants, because strain SKN was isolated from a sewage plant and an independent enrichment culture also yielded a similar organism (Schleheck, unpublished data).

The specific rate of degradation of saccharin in the growing culture [1.1 mkat $(kg\ protein)^{-1}$] was lower than the specific activity of cells in suspension [≤ 3.8 mkat $(kg\ protein)^{-1}$] (Fig. 2a). Therefore, the putative multi-component saccharin dioxygenase involved in the conversion of saccharin to catechol (Fig. 3) is highly active in harvested cells, but its activity is lost when the cells are disrupted or permeabilized. The activity of the *ortho* ring cleavage reaction in whole cells [8 mkat $(kg\ protein)^{-1}$] and in cell extracts [3.3 mkat $(kg\ protein)^{-1}$], and the activity of the muconate lactonizing enzyme in cell extracts [1.1 mkat $(kg\ protein)^{-1}$] are adequate to explain the growth rate.

Several possible modes of attack on saccharin can be postulated (e.g. Fig. 1), but the experiments show oxygenation as the first reaction, and the data on oxygen uptake indicate only two oxygenation reactions. The first is by a putative saccharin dioxygenase and the second is the *ortho* ring cleavage reaction [EC 1.13.11.1], catalyzed by an enzyme with a narrow substrate range (Harwood and Parales 1996). There is no indication of a *meta* cleavage (with the wider substrate range that would allow). Therefore, both substituents on the saccharin aromatic ring must be removed by saccharin dioxygenase. Precedents for this suggestion are the two- and three-component 2-halobenzoate dioxygenases, which generate catechol (Fetzner et al. 1992; Romanov and Hausinger 1994), and the physiological data in Fig. 2a imply the presence of a multi-component oxygenase. It seems likely that saccharin dioxygenase releases the [–SO_2–NH–CO–]-moiety of the saccharin heterocyclic ring during the reaction, but it is still unclear which biological reactions are involved in generating the sulfate and ammonium ions detected after growth (Fig. 2b).

The stoichiometric formation of catechol from saccharin (Fig. 3) makes the simultaneous presence of a second pathway unlikely. An initial reaction on the heterocyclic ring via hydrolysis of the amide bond [see EC 3.5.1.-] or of the sulfamate bond [see EC 3.10.1.-] (Fig. 1) was not detected, no putative pathway intermediate (2-sulfobenzoate, 2,3-dihydroxybenzoate or 3-sulfocatechol) was subject to metabolism, and the required *meta* cleavage (see Hansen et al. 1992; Junker et al. 1994b) was absent. An initial attack on the unsubstituted carbon atoms of the saccharin ring (Fig. 1) would lead to very complex intermediates, and, as with an initial monooxygenase of the type known in assimilative desulfonation (see Kahnert et al. 2000), would probably require at least three oxygenases in the pathway.

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