

RESEARCH ARTICLE

Isolation, identification and characterization of human intestinal bacteria with the ability to utilize chloramphenicol as the sole source of carbon and energy

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Abstract

Five aerobic intestinal bacterial strains that utilized chloramphenicol (CAP) as sole carbon and energy source were isolated from fecal samples collected from healthy volunteers. Based on 16S rRNA gene sequence analysis, four of the five strains were identified as *Klebsiella pneumoniae* and one as *Escherichia fergusonii*. The degradation rate of strain I-10-CHL (*E. fergusonii*) varied with the initial concentration of CAP. The pH value also had an effect on the degradation rate of CAP and bacterial growth. A pH of 6.5 was optimal for CAP degradation and growth of strain I-10-CHL (*E. fergusonii*). In mixed substrate batch cultivations, where CAP was one of the components, glucose, acetamide and CAP were utilized simultaneously. The presence of glucose and acetamide increased the growth and substrate degradation rates of CAP. During incubation with *E. fergusonii* cells, reduction intermediates (1-*p*-nitrophenyl-2-amino-1,3-propanediol) were observed. The strains capable of utilizing CAP were CAP-susceptible, which indicates that the mechanism of CAP assimilation by the strains is independent on that of CAP resistance. The role of this CAP reduction pathway in the physiology of *K. pneumoniae* and *E. fergusonii* is unknown.

Introduction

The *p*-nitroaromatic antibiotic chloramphenicol (CAP) has been used extensively for the treatment of life-threatening infections due to *Haemophilus influenzae* and *Neisseria meningitidis*, and its mechanism of action is the inhibition of protein synthesis (Smith *et al.*, 2007). CAP was originally derived from the bacteria *Streptomyces venezuelae*, isolated by David Gottlieb, and introduced into clinical practice in 1949, under the trade name Chloromycetin. It was the first antibiotic to be manufactured synthetically on a large scale (Brock, 1961). As a result of its relatively simple structure, a large number of modifications of this antibiotic have been prepared and tested.

During the past five decades, utilization of CAP by a few pure strains has been reported. Isolation of a pure bacterial strain, which was identified as *Streptomyces* sp., was first reported by Abd-El-Malek *et al.* (1961). A recent study has found that CAP was a substrate for a novel

nitroreductase pathway in *H. influenzae* (Smith *et al.*, 2007). A further recent study demonstrated that a diverse group of bacteria, including intrinsically resistant microbes (for example, Pseudomonadales and Burkholderiales), could subsist on and presumably catabolize antibiotics as a sole carbon source (SCS) (Dantas *et al.*, 2008). In recent study, Barnhill *et al.* (2010) assessed this subsistence phenotype in multiresistant and antibiotic-sensitive *Salmonella* isolates.

In our preliminary study of intestinal bacteria subsisting on CAP, eight antibiotics besides CAP were investigated: penicillin, carbenicillin, ampicillin, gentamicin, kanamycin, streptomycin, tetracycline and vancomycin. Taking into account that the structure of CAP molecular is relatively simple, and that its research background is more profound, we primarily took CAP as the focus for the further study. In present research, we report on the bio-degradation of CAP by five aerobic intestinal bacterial strains, the effects of environmental factors on

CAP-degradation and bacterial growth, and the identification of metabolites.

Materials and methods

Growth media

All liquid media used for isolating bacteria capable of subsisting on CAP were made by dissolving 1 g L⁻¹ of CAP into SCS media (Dantas *et al.*, 2008) containing 5 g (NH₄)₂SO₄, 3 g KH₂PO₄, 0.5 g MgSO₄·7H₂O, 15 mg EDTA, 4.5 mg ZnSO₄·7H₂O, 4.5 mg CaCl₂·2H₂O, 3 mg FeSO₄·7H₂O, 1 mg MnCl₂·4H₂O, 1 mg H₃BO₃, 0.4 mg Na₂MoO₄·2H₂O, 0.3 mg CuSO₄·5H₂O, 0.3 mg CoCl₂·6H₂O and 0.1 mg KI per liter water. The pH was adjusted to 5.5 using HCl, and the media was sterilized through a 0.22-µm filter. Solid medium was prepared by adding 15 g agar per liter of liquid SCS media followed by autoclaving before adding the antibiotic.

Luria–Bertani (LB) broth media was used as the seed culture media and contained 5 g yeast extract, 10 g NaCl and 10 g of tryptone in 1 L of water. The pH was adjusted to 7.0 using HCl, and the media was sterilized through a 0.22-µm filter.

Fecal sample collection

The fecal samples were collected from four healthy volunteers aged 20–75 years from the same local community. Samples were submitted to the investigators within 15 h of collection, sealed with parafilm, and transported and stored on ice.

Isolation and identification of intestinal bacteria capable of subsisting on CAP

Initial fecal microbial inocula were prepared in minimal media containing no carbon, and inoculated into SCS-CAP media (corresponding to approximately 125 mg of dissolved fecal material in 5 mL of media). To significantly reduce the transfer of residual alternative carbon sources present in the original inocula, samples were passaged (2.5 µL) into fresh SCS-CAP media (5 mL) two additional times after 7 days of growth. Clonal isolates from the liquid cultures were obtained by plating cultures out on an SCS-CAP agar medium and the resulting single colonies were picked and restreaked on SCS-CAP plates. Three colonies each were then inoculated into fresh SCS-CAP liquid media (5 mL) to confirm the clonal phenotype. Final culture growth was recorded after 1 month's incubation without shaking at 37 °C and cultures with at least 10⁸ cells mL⁻¹ were assayed as positive growth. Five resultant isolates were characterized by standard

bacteriological methods and by amplification and sequencing of the 16S rRNA gene. MEGA 5.0 software was used for sequence alignment and generation of phylogenetic trees.

Testing for antimicrobial susceptibility

Five isolates were assayed for susceptibility to 14 antibiotics using a disk diffusion method. The results were interpreted in accordance with the standards of the National Committee for Clinical Laboratory Standards, where available (CLSI 2010).

Screening and degradation experiments

Initially, five isolates were screened for possible CAP-degrading activity. The bacterial growth and the biodegradation of CAP were monitored in 40 mL of a working medium containing 20 µg mL⁻¹ of CAP as sole source of carbon and energy. Cultures were incubated at 37 °C at 180 r.p.m. on a rotary shaker. The concentration of residual CAP and the bacterial growth (colony-forming units) were measured and evaluated after 13 days. For strain I-10-CHL (*Escherichia fergusonii*), the experiments were performed under the same conditions, but with more frequent monitoring and with an initial bacterial concentration adjusted to 1 × 10⁷ CFU mL⁻¹. The working medium was the SCS medium containing 20 µg mL⁻¹ CAP.

Effects of initial concentrations of CAP on growth and CAP degradation of strain I-10-CHL

To each flask filled with 35 mL minimal salt medium, 0.10, 0.20, 0.30, 0.40, 0.50 and 0.60 mL CAP ethanol solution with a concentration of 20 g L⁻¹ was added and then inoculated with 5-mL suspension of strain I-10-CHL, which made the initial concentration of CAP in the SCS medium approximately 25, 50, 75, 100, 125 and 150 mg L⁻¹, respectively. Cultures were incubated at 37 °C at 180 r.p.m. on a lucifugal rotary shaker. The concentration of residual CAP and the bacterial growth (colony-forming units) were measured and evaluated after 7 days. The control group was set at the same time.

Effect of pH value on growth and CAP degradation of strain I-10-CHL

A 5-mL suspension of strain I-10-CHL was inoculated into each flask filled with 35 mL SCS-CAP media of different set pH values: the concentration of CAP in the SCS media was 25 mg L⁻¹; prior to sterilization, the pH value of each minimal salt media was adjusted to 5.0, 5.5,

6.0, 6.5, 7.0, 7.5 and 8.0, respectively. Cultures were incubated at 37 °C at 180 r.p.m. on a lucifugal rotary shaker. The concentration of residual CAP and the bacterial growth (colony-forming units) were measured and evaluated after 7 days. The control group was set at the same time.

Effect of other carbon sources on the growth and CAP degradation of strain I-10-CHL

The effects of the four other carbon sources, with some functional groups similar to that of CAP, on the growth and CAP degradation of strain I-10-CHL were determined by inoculating LB-grown cells into a mineral medium containing benzoic acid, acetamide, glucose, trichloroacetic acid (TDA) and salicylic acid with CAP.

In each flask filled with 35 mL mineral medium containing 25 µg mL⁻¹ CAP, glucose, benzoic acid, acetamide, TDA and salicylic acid were added and then inoculated with a 5-mL suspension of strain I-10-CHL [the concentration of CAP in the SCS media was 25 mg L⁻¹ and the concentration of additive carbon source reached 0.01% (w/v)]. Cultures were incubated at 37 °C at 180 r.p.m. on a lucifugal rotary shaker. The concentration of residual CAP and the bacterial growth (colony-forming units) were measured and evaluated after 7 days. The control was set at the same time.

Analytical methods

UV-Vis analysis

Chemical and physical properties of the metabolites were analyzed with SHIMADZU UV-2450. The scanning wavelength range was 190–400 nm, scanning interval 0.5 nm, and the scanning speed was low.

High performance liquid chromatography

Levels of CAP and metabolites were analyzed by high performance liquid chromatography (HPLC) using a monochromatic UV detector set at 275 nm. The mobile

phase was acetonitrile : water 20 : 80 (v/v), with a gradient (30 min) to acetonitrile : water 50 : 50 (v/v). Aliquots of 20 µL were injected onto a C-18 column (4.6 × 250 mm, 5 µm particles) at room temperature. The flow rate was 0.8 mL min⁻¹.

LC-MS/MS

Structure characterization of CAP and metabolites was detected by liquid mass spectrometry instrument.

For mass spectrometry, a Waters Maldi synapt Q-TOF mass spectrometer operated in positive electrospray ionization mode was used: capillary voltage: 3.5 kV, cone voltage: 45 V, source block temp: 100 °C, desolvation temp: 300 °C, desolvation gas flow: 500 L h⁻¹, cone gas flow (L h⁻¹): 50 L h⁻¹, collision energy (ev): 6 V, mass range: 600–3000 m/z, detector voltage: 1600 V. The ultra performance liquid chromatographic conditions were as follows. Levels of CAP and metabolites were analyzed by ultra performance liquid chromatography (Waters ACQUITY UPLC) using a monochromatic UV detector (Waters ACQUITY PDA) set at 280 nm. The mobile phase was 0.1% methanoic acid : acetonitrile (70 : 30 v/v) with a gradient (200 min) to 100% acetonitrile. Aliquots of 1 µL were injected onto a C-4 column (2.1 × 100 mm, 1.7 µm particles) at 45 °C. The flow rate was 0.3 mL min⁻¹. The data acquisition was in the multichannel analysis mode using MICROMASS MASSLYNX 3.4 software.

Results and discussion

Isolation of intestinal bacteria capable of subsisting on CAP

Several bacterial strains, which were potentially able to use CAP as a sole source of carbon and energy, were isolated from SCS-CAP plates. Based on their morphology, five of these isolates (three from fecal samples of the elderly and two from samples from the younger population) were picked and initially screened for their real CAP-degrading activity (Table 1). Degradation rates were analyzed by HPLC and bacterial density was measured by a UV-Vis spectrophotometer. Under the conditions as described in

Table 1. Physiological features and CAP-degrading rates of five different isolates

Strains	Colonial morphology	Gram stain	Speed of growth	CAP-biodegradation rate after 13 days' cultivation (%)
I-11-CHL-1	Tiny, pale yellow, smooth surface, subtransparent	G ⁻	Fast	10.5
II-3-CHL-1	Tiny, pale yellow, smooth surface, subtransparent	G ⁻	Fast	15.7
I-10-CHL	Tiny, ivory, smooth surface, subtransparent	G ⁻	Slow	45.5
II-2-CHL-3	Tiny, pale yellow, smooth surface, subtransparent	G ⁻	Slow	4.1
I-7-CHL-1	Tiny, pale yellow, smooth surface, subtransparent	G ⁻	Fast	7.3

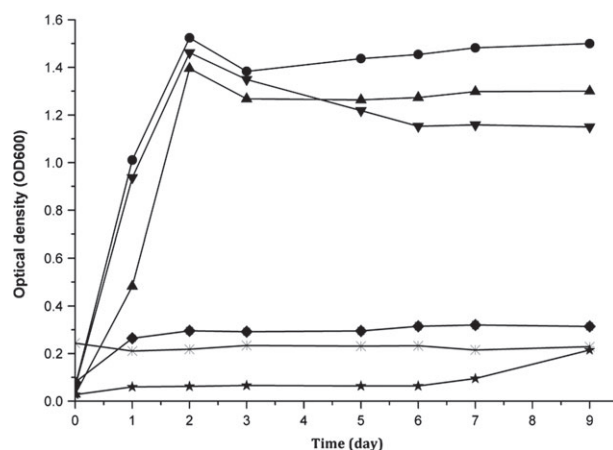


Fig. 1. Growth of the five strains with CAP-degrading capability in the SCS media. (◆) II-2-CHL-3; (●) I-11-CHL-1; (▲) I-7-CHL-1; (▼) II-3-CHL-1; (★) I-10-CHL; (X) Control. The bacterial growth was monitored in 40 mL of a working medium containing 20 mg L⁻¹ CAP as sole source of carbon and energy. Cultures were incubated at 37 °C at 180 r.p.m. on a rotary shaker.

Screening and degradation experiments, strain I-10-CHL, whose 13-day degradation rate reached 45.5%, seemed the most efficient for the degradation and was selected for further study.

Although strain I-10-CHL had the highest CAP-degrading rate, it demonstrated a significant lag phase of approximately 6 days, which was much longer than that observed with any other strain (Fig. 1).

Identification and phylogenetic analysis of intestinal bacterial capable of subsisting on CAP

Five clonal isolates were identified by amplifying and sequencing the 16S rRNA gene with the universal bacterial 16S rRNA gene primers. MEGA 5.0 software was used for sequence alignment and generation of phylogenetic trees.

The 16S rRNA gene sequences of the five clonal isolates showed similarity to 16S rRNA gene sequences from members of the genera *Klebsiella* and *Escherichia*. The results of BLAST in the NCBI are presented in the Table 2. A dendrogram illustrating the results of the 16S rRNA gene analysis using MEGA 5.0 is shown in Fig. 2. These results again indicate

that the strain I-10-CHL belongs within the *E. fergusonii* group. Overall, the strain I-10-CHL sequence had the greatest similarity to enterobacter-like isolates within this group which are clinically relevant pathogens (Sommer *et al.*, 2009).

Organisms in the *Klebsiella* and *Escherichia* genera typically have large genomes of approximately 4–7 megabases, which has been proposed as positively correlated with their metabolic diversity and multiple antibiotic resistance (Projan, 2007). However, this is the first report on CAP degradation by species in the genera of *Klebsiella* and *Escherichia*.

Testing for antimicrobial susceptibility

Five isolates were assayed for susceptibility to 14 antibiotics using a disk diffusion method. The results were interpreted in accordance with the standards of the National Committee for Clinical Laboratory Standards, where available. The results for inhibition zone diameters by 14 antibiotics disc diffusion can be seen in Table 3.

The five isolates were subjected to a CAP disc diffusion test using a 30-μg disc. A 0.5 McFarland standard suspension of the isolate was made and lawn culture done on a Mueller-Hinton broth Agar (MHA) plate. Plates were incubated at 37 °C for 18 h and zone diameters were measured. An inhibition zone diameter < 12 mm was reported as CAP-resistant and more than 18 mm was considered sensitive. Each batch of drug sensitive test was performed using strain ATCC 25922 as the quality control strain.

CAP sensitivity was observed in all of the five isolates that subsisted on this antibiotic. This indicates that the mechanism of CAP resistance does not appear to be related to the subsistence activity. Recently, Barnhill *et al.* (2010) assessed antibiotic subsistence phenotype in multi-resistant and antibiotic-sensitive *Salmonella*. They proved that subsistence and resistance did not appear to be equivalent, as been demonstrated by Dantas *et al.* (2008).

Degrading ability of strain I-10-CHL

The assimilation of CAP by *E. fergusonii* was investigated in more detail. Figure 3 presents a typical result. The initial CAP concentration was set at approximately 55 mg L⁻¹. After 8 days, approximately 40% of the CAP

Table 2. Identification of five CAP-degrading strains

CAP-degrading strains	The closet gene identities in Genbank	NCBI accession nos of CAP-degrading strains	16S rRNA gene sequence similarity (%)
I-11-CHL-1	<i>Klebsiella pneumoniae</i> strain BCH1	JQ838151	99
I-10-CHL	<i>Escherichia fergusonii</i> strain G7	JQ838153	99
I-7-CHL-1	<i>Klebsiella pneumoniae</i> KCTC 2242	JQ838155	99
II-2-CHL-3	<i>Klebsiella</i> sp. NCCP-141	JQ838154	99
II-3-CHL-1	<i>Klebsiella</i> sp. NCCP-141	JQ838152	99

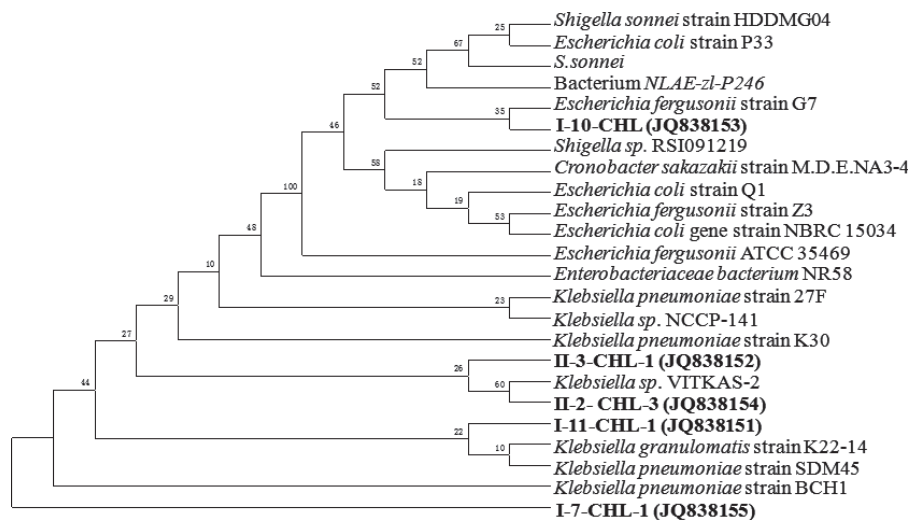


Fig. 2. Phylogenetic tree based on the 16S rRNA gene sequences of the candidate phyla *Escherichia*, *Klebsiella* and other relative species. Strain I-10-CHL and other isolates from this study are highlighted in bold. The tree was constructed in MEGA 5.0 software.

Table 3. The antibiotic-resistance spectrum of five CAP-degrading strains

Drug*	Zone of inhibition (mm)					Results†				
	I-11-CHL-1	II-3-CHL-1	I-7-CHL-1	II-2-CHL-3	I-10-CHL	I-11-CHL-1	II-3-CHL-1	I-7-CHL-1	II-2-CHL-3	I-10-CHL
CIP	29.2	32.7	27.8	30.0	32.7	S	S	S	S	S
FLX	25.2	24.6	23.4	23.6	23.4	S	S	S	S	S
CEP	22.5	19.2	16.7	21.7	22.5	S	S	S	S	S
CAR	6.6	6.6	6.6	6.6	6.6	R	R	R	R	R
GEN	22.5	24.3	23.9	21.9	24.3	S	S	S	S	S
AMP	6.6	6.6	6.6	6.6	6.6	R	R	R	R	R
STR	22.4	22.5	6.6	24.4	22.5	S	S	R	S	S
TET	19.6	19.8	10.2	18.6	19.8	S	S	S	S	S
NAL	23.5	22.2	22.0	22.0	22.2	S	S	S	S	S
CHL	27.3	24.4	24.2	29.5	24.2	S	S	S	S	S
NET	27.3	24.4	24.2	23.8	24.2	S	S	S	S	S
IPM	21.8	21.9	20.9	23.7	21.8	S	S	S	S	S
SUL	24.4	6.6	23.7	6.6	6.6	S	R	S	R	R
KAN	23.8	25.4	25.1	24.3	25.4	S	S	S	S	S

*CIP, ciprofloxacin; FLX, fleroxacin; CEP, cephaloridine IV; CAR, carbenicillin; GEN, gentamicin; AMP, ampicillin; STR, streptomycin; TET, tetracycline; NAL, nalidixic acid; CHL, chloramphenicol; NET, netilmicin; IPM, imipenem; SUL, sulfagan; KAN, kanamycin.

†The results were interpreted in accordance with the standards of NCCLS, when available. S, susceptible; R, resistance; I, intermediary. Each batch of drug-sensitive tests was performed using strain ATCC 25922 as the quality control strain.

had disappeared from the culture medium and after 12 days, approximately 84% of CAP had been degraded. In the uninoculated control, the CAP concentration did not decrease, remaining at the original concentration of approximately 55 mg L⁻¹.

The growth of *E. fergusonii* during the assimilation is also presented in Fig. 3. Maximal growth rate was observed after 8 days of incubation (8–12 days), during which period most of the CAP had degraded. This result indicates that the strain I-10-CHL is able to utilize CAP as the sole carbon and energy source.

Effect of initial concentration of CAP

In the biodegradation research component, the initial concentration played a significant role in the degradation rate. As CAP itself demonstrates bacteriostasis to some extent, the effect of its concentration on bacterial growth and degradation rate could be followed. Referring to current research on CAP, we studied the degradation and growth of strain I-10-CHL under different CAP concentrations of 25, 50, 75, 100, 125 and 150 mg L⁻¹ in SCS media. The bacterial density was calculated using the

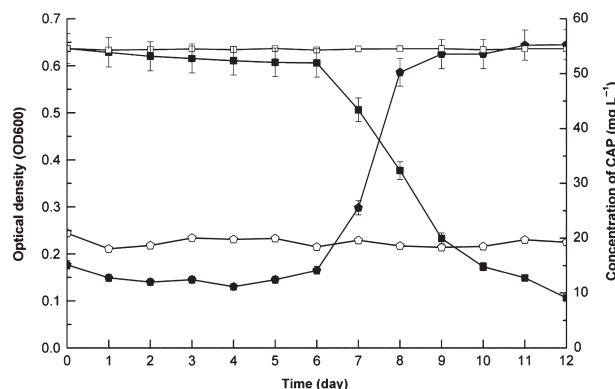


Fig. 3. Growth and CAP-biodegradation by strain I-10-CHL. (●) Growth and (■) CAP-biodegradation with CAP (55 mg L⁻¹); (○) growth and (□) CAP-biodegradation without CAP. The basal medium was SCS medium.

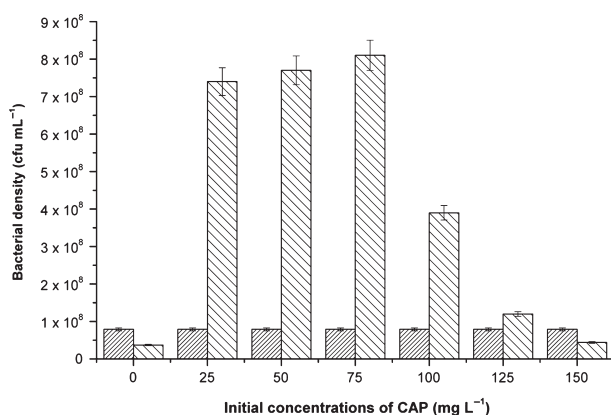


Fig. 4. Effect of the initial CAP concentration on the growth of strain I-10-CHL. (■) Bacterial density at the start and (▨) after 7 days' cultivation. The working medium was SCS medium containing CAP as the sole source of carbon and energy. Cultures were incubated at 37 °C at 180 r.p.m. on a rotary shaker.

plate counting method and the residual concentration of CAP was measured by HPLC. The results are showed in Figs 4 and 5.

It was observed that the average growth of the biomass under CAP concentrations in the range of 25–75 mg L⁻¹ was higher than under CAP concentrations in the range of 100–150 mg L⁻¹. The largest growth of the biomass was observed at 75 mg L⁻¹. This shows that substrate inhibition may occur at higher CAP concentrations, as CAP functions as antibacterial at high concentrations rather than as the source of carbon and energy.

The CAP degradation rate of strain I-10-CHL was negative correlated with CAP concentration. As a result of the antibacterial effect of CAP, the growth of the biomass did not substantially increase as the CAP concentrations increased. Moreover, the amount of CAP utilized by

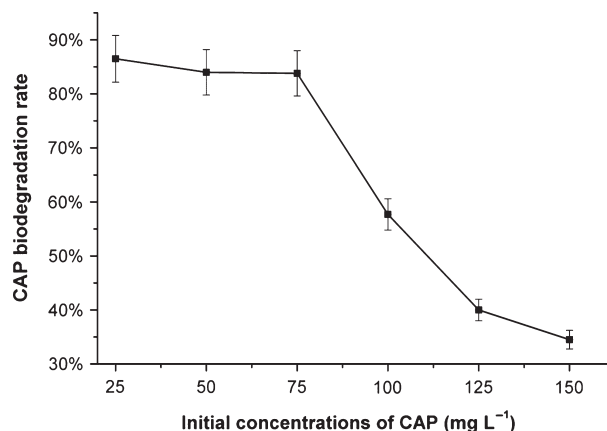


Fig. 5. Effect of the initial concentration of CAP on CAP-biodegradation by strain I-10-CHL. The working medium was SCS medium containing CAP as the sole source of carbon and energy. Cultures were incubated at 37 °C at 180 r.p.m. on a rotary shaker.

strain I-10-CHL changed little. Therefore, the degradation rate of CAP was at a low level under higher concentrations of CAP, whereas it was at a high level under low concentrations of CAP, the antibiotic at low concentrations being more available as the carbon and energy source for the bacteria. The highest CAP degradation rate of 86.5% was observed at the concentration of 25 mg L⁻¹. As result, if the concentration of CAP in the natural environment is lower, bacteria will be able to utilize CAP more efficiently as the carbon and energy source.

Based on the microbroth dilution test for CAP susceptibility, the MIC of the strain I-10-CHL was 16 mg L⁻¹. However, the growth of the strain I-10-CHL in SCS media began to be reduced when the CAP concentration reached 75 mg L⁻¹. This indicates that CAP-resistance is not equal to CAP-subsistence, since the former phenotype occurs during short period of incubation in Mueller–Hinton Broth media, and the latter during long period of incubation in SCS media.

Effect of pH value

The pH value has a marked influence on microbial growth. The main effect of the pH value is to induce a change in the cell membrane charge (the bacterial surface usually has a negative charge) as well as the degree of ionization of nutritional substances, which could influence the utilization of nutrition by the bacteria (Guy *et al.*, 2004).

In this study, we determined the CAP degradation rate of strain I-10-CHL under seven different pH values, 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and 8.0, during a period of 7 days. Through

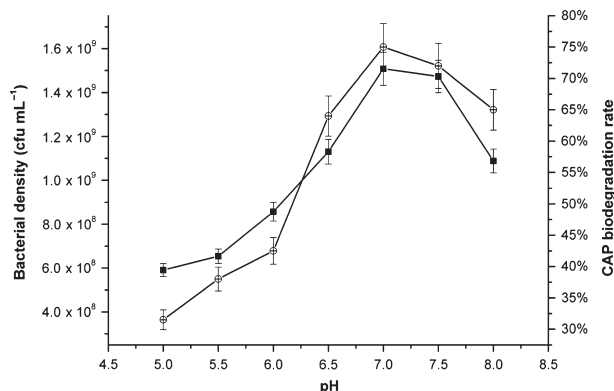


Fig. 6. Effect of pH on growth of strain I-10-CHL and CAP-biodegradation by strain I-10-CHL. (■) Bacterial density after 7 days' cultivation; (○) CAP degradation rate after 7 days' cultivation. The working medium was SCS medium containing 25 mg L⁻¹ CAP as the sole source of carbon and energy. Cultures were incubated at 37 °C at 180 r.p.m. on a rotary shaker.

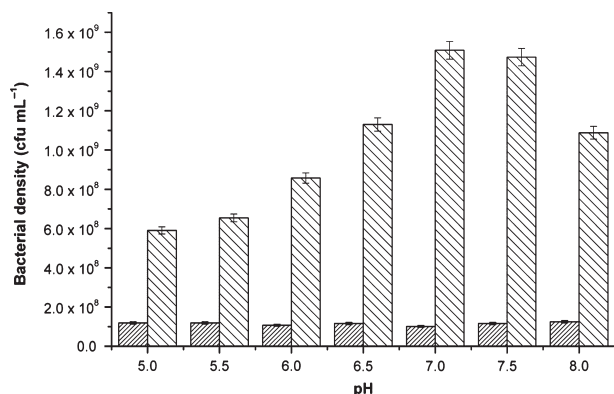


Fig. 7. Effect of pH on growth of strain I-10-CHL. (■) Bacterial density at the start and (▨) after 7 days' cultivation. The working medium was SCS medium containing 25 mg L⁻¹ CAP as the sole source of carbon and energy. Cultures were incubated at 37 °C at 180 r.p.m. on a rotary shaker.

the method of plate dilution coating, we calculated the bacterial density before and after 1 week. As shown in Figs 6 and 7, the strain I-10-CHL had a slightly higher growth rate at pH 6.5–8.0 than at pH 5.0–6.0. In particular, with a pH of 7.0, the strain I-10-CHL demonstrated maximal bacterial density and CAP degradation rates.

Effect of additives

The response of the CAP-adapted organism to some compounds with similar functional groups to CAP was studied by using these compounds in combination with CAP. Strain I-10-CHL was cultivated in minimal salt media containing 25 mg L⁻¹ CAP with additives of 0.1%

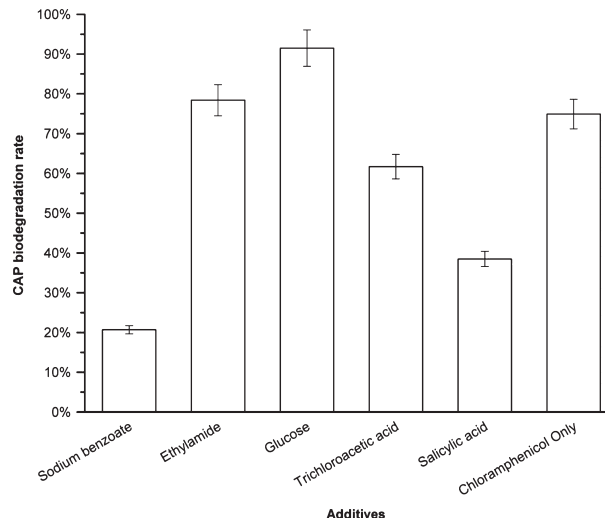


Fig. 8. Effect of additional carbon sources on CAP-biodegradation by strain I-10-CHL. The working medium was SCS medium containing 25 mg L⁻¹ CAP and 0.01% (w/v) of another carbon source. Cultures were incubated at 37 °C at 180 r.p.m. on a rotary shaker.

glucose, acetamide, sodium benzoate, TDA and salicylic acid. The degradation rate of CAP was measured by HPLC (Fig. 8). The strain I-10-CHL growth of the biomass with the minimal salt media and additives of different carbon sources was measured using the plate counting method (Fig. 7).

TDA and salicylic acid did not support the growth of the organism and lowered the CAP degradation rate. Thus strain I-10-CHL is specific for the substrate, which indicates the specific nature of CAP-transporting and/or -degrading enzymes. Acetamide not only increased the biomass yield of strain I-10-CHL but also promoted the CAP degradation rate. The results indicate that acetamide with the amide group may induce more production of amidohydrolase, which facilitated the reduction of CAP. Although sodium benzoate increased the growth of the biomass of strain I-10-CHL, it lowered the utilization rate of CAP (Fig. 9).

Analysis of products of CAP metabolism by UV absorbance spectrum

Examination of CAP culture supernatants collected during incubation of CAP with strain I-10-CHL indicated the conversion of CAP into at least two intermediate compounds before accumulation of the equilibrium-favored product (Fig. 10).

The UV absorption spectrum of authentic CAP has two peaks with maximum absorbance at 201 and 278 nm, respectively. Within 5 days, the height of the peaks of 201 and 278 nm had both decreased. By day 6,

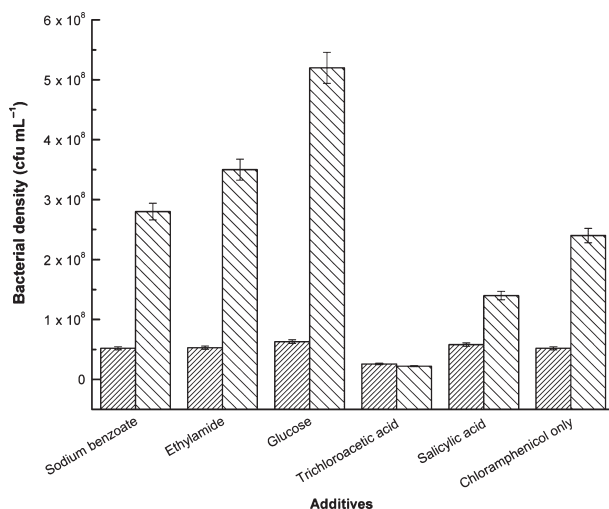


Fig. 9. Effect of additives on growth of strain I-10-CHL. (■) Bacterial density at the start and (▨) after 7 days' cultivation. The working medium was SCS medium containing 25 mg L⁻¹ CAP and 0.01% (w/v) of another carbon source. Cultures were incubated at 37 °C at 180 r.p.m. on a rotary shaker.

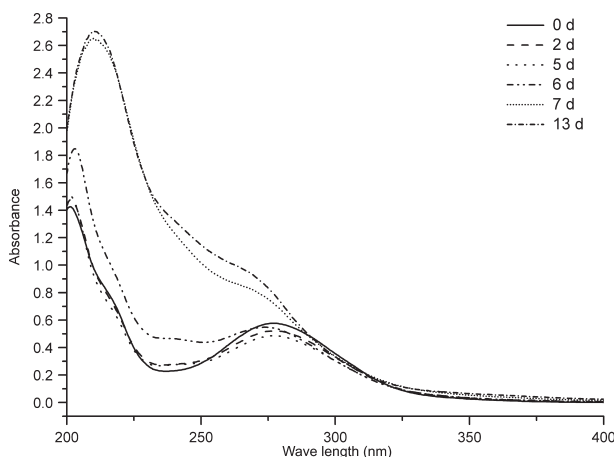


Fig. 10. UV scan spectrum of supernatant samples obtained at several different time points. Strain I-10-CHL was incubated in SCS medium with CAP at 37 °C for 13 days, and aliquots were taken and centrifuged to collect the culture supernatants at the times indicated (D0 = zero time, D2 = 2 days, etc.). The absorbance between 193 and 400 nm was determined for each aliquot with the peak absorbance indicated.

the peak of 278 nm shifted to 274 nm, and became weaker, while the peak of 201 nm moved to 203 nm and became sharper. Over the next day, the peak of 203 nm shifted to 210 nm, developing into a wide peak. The peak of 274 nm disappeared, and became a shoulder of the peak of 210 nm. The height of this peak was greatest at 13 days and the shoulder was more apparent. This was considered to be the final product, as there was no fur-

ther change in the spectrum after 2 days of incubation (data not shown). It is noteworthy that the samples were derived from culture supernatants, indicating that if the reaction occurred intracellularly, the intermediates diffused readily in and out of the cell, and the final product was extracellular (Smith *et al.*, 2007).

LC-MS/MS analysis of metabolites

The fermentation liquid supernatant samples collected at day 0 and day 13 were detected by HPLC. When the spectra of the two samples were compared, a new peak of a possible metabolite was observed in the spectrum of the final fermentation supernatant.

The study compared authentic CAP with the intermediate metabolite. As described below, the data were most consistent with reduction of COCHCl₂ to yield 1-*p*-nitrophenyl-2-amino-1,3-propanediol (Fig. 11). The hypothesized pathway for this compound is depicted in Fig. 12.

One unique aspect of the compound CAP is that it contains two chlorine atoms, thus giving rise to unique isotopic patterns (Fig. 11a). However, no such isotope cluster is observed in Fig. 11b, implying that two chlorine atoms are reduced from the parent CAP. Both spectra of parent CAP molecular and metabolite molecular exhibit the loss of COCHCl₂, CH₂OH and H₂O, resulting in a common *m/z* 165 base peak, illustrating that there is a common backbone in both compounds. MS/MS spectra of the *m/z* 213 in the metabolite spectra yielded fragment ions *m/z* 195, 165 corresponding to the loss of H₂O, C₂HOC₂Cl₂ + CH₂OH + H₂O from the metabolite (data not shown). These data confirm 1-*p*-nitrophenyl-2-amino-1,3-propanediol as the observed species (Brock, 1961; Shin, 1971; Zeegers & Gibella, 1997).

Due to lack of authentic substances, we were unable to analyze realistic metabolic products. Comparing our results with data from other research, we suggest that the possible biodegradation pathway of CAP is as follows: CAP is initially hydrolyzed into 1-*p*-nitrophenyl-2-amino-1,3-propanediol as well as dichloroacetamide, and then converted into CO₂ + H₂O + NH₄⁺ + Cl⁻.

According to the literature, the microorganisms subsisting on antibiotics are all culturable, the majority being bacteria and the minority mould. Such microbes exist in natural environments such as soil, lake sediment and poultry meat products. This report is the first to demonstrate that *Escherichia* spp. and *Klebsiella* spp. are capable of subsisting on the antibiotic CAP which was isolated from the fecal samples collected from healthy individuals. From a clinical perspective, subsistence should be unnecessary in a host, due to the abundance of carbon sources available. Nonetheless, the findings of this study raise important issues (Barnhill *et al.*, 2010).

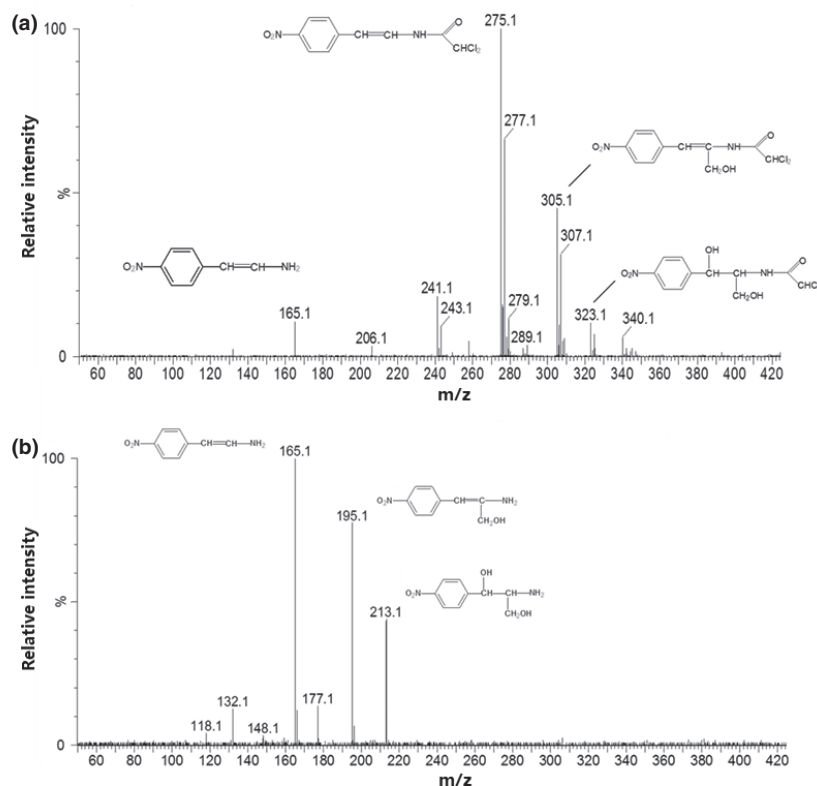


Fig. 11. Mass spectra of authentic CAP (a) and intermediary (b).

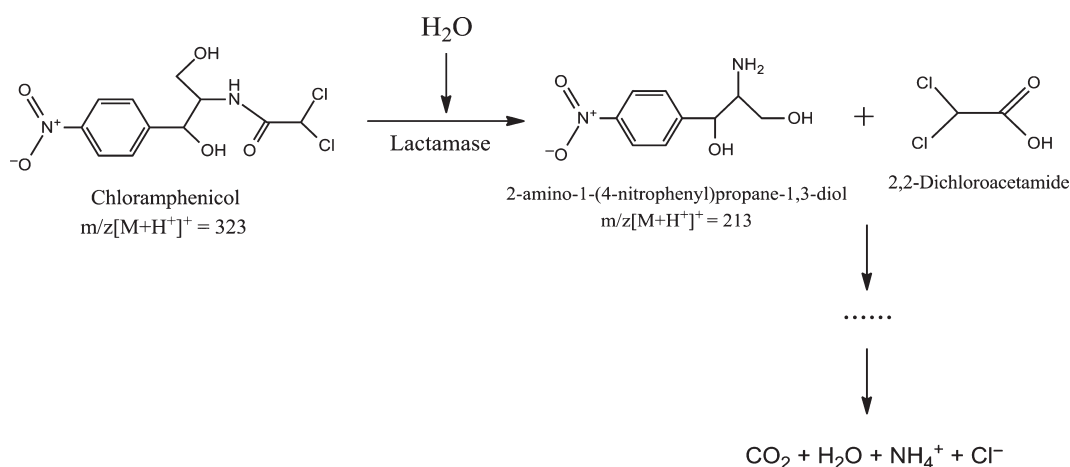


Fig. 12. The hypothesized initial degradation of CAP by strain I-10-CHL.

First, many oral antibiotics can interfere with the intestinal flora, and the intestinal flora can also change or modify the antibiotic molecules. Such antibiotics include azo sulfa-drugs, CAP and succinylsulfathiazole. With continuously exposed to exogenous compounds through oral administration, the intestinal flora will suffer from

selective pressure, which results in the propagation of specific microbes minimizing antibiotics and other xenobiotics in the intestinal gut.

Secondly, it is reported that CAP can be absorbed rapidly from the gut. During enterohepatic circulation, two microbial metabolites are formed. The major product is

the deacetylated form (which also has no antibacterial action), but there is also reduction of the nitro group, giving an arylamine (Williams *et al.*, 1971). How the intestinal bacteria subsisting on CAP affect the host physiology is uncertain. Based on the metabolism pathway hypothesized above, we infer that under some conditions, such specific intestinal flora may have a potential impact on the fate of CAP *in vivo*.

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