

ATR-FTIR spectroscopic investigation of *E. coli* transconjugants β -lactams-resistance phenotype

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Abstract Hyphenation of attenuated total reflection Fourier transform infrared spectroscopy and cluster analysis has been used to characterise a susceptible *Escherichia coli* K12 strain and the transconjugants TEM-1, TEM-2, TEM-3, SHV-2, SHV-3, SHV-4. A good discrimination of the susceptible strain from the transconjugants was obtained. Although a limited success was achieved in the differentiation of SHV and TEM phenotypes in general, results obtained with TEM-2 and SHV-3 were convincing. Spectral differences observed are ascribed to the global effects of the conjugation process, particularly their repercussions in the nucleic acids and carbohydrate absorbing regions, rather than to β -lactamase point-mutations.

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Key words: *Escherichia coli* K12; TEM transconjugant; SHV transconjugant; ATR-FTIR spectroscopy; Cluster analysis

1. Introduction

Most strains of *Escherichia coli* are very susceptible to many β -lactams. However, clinical isolates can be resistant to β -lactams by high-level production of a chromosomally encoded cephalosporinase or by production of plasmid-mediated β -lactamases [1]. These strains are responsible for serious nosocomial infections in French hospitals since 1985 and thus knowledge of the main resistance patterns of *E. coli* isolates to β -lactam agents should help in the choice of therapeutic strategies. The characterisation of TEM and SHV phenotypes can be performed by studying the biochemical properties of β -lactamases (extended-spectrum or extended broad-spectrum β -lactamases) by isoelectric focusing, by determination of substrate and inhibition profile [2,3] and by hybridisation or nucleotide sequencing [4]. However, access to a new tool that enables rapid and straightforward characterisation of various TEM and SHV phenotypes seems of importance. In this work, we propose ATR-FTIR spectroscopy as such a means.

FTIR methods in general are non-destructive and have molecular 'fingerprinting' capabilities. In transmission mode, they have proved to be successful in bacterial classification at different levels of taxonomic discrimination without any preselection of strains by other taxonomic criteria [5], in studying the effect of growth medium [6] on the ultrastructures of microbials. The originality of the ATR technique compared to transmission or diffuse reflectance-absorbance methods is that

it offers several advantages. For example, the problems, mainly baseline drifts, associated with these methods and pointed out by other investigators [7,8] can be circumvented. Its use for bacterial studies is simpler since no suspension or dehydration of the sample is required. Recently, associated with chemometrics methods, it was successfully used to discriminate isogenic strains of *P. aeruginosa* with varying degrees of resistance to imipenem [9].

By applying cluster analysis to a set of ATR-FTIR spectra recorded under well-established conditions, we wish to address the following questions. Is it possible to discriminate (i) the SHVs and TEMs from *E. coli* C600 K12-susceptible strain, (ii) the SHVs, (iii) the TEMs, and (iv) the TEMs from the SHVs? To do so, we have investigated a susceptible *E. coli* C600 K12 strain and six of the transconjugants: TEM-1, TEM-2, TEM-3, SHV-2, SHV-3 and SHV-4. Furthermore, the spectral characteristics of one member of each class have been analysed in more detail in order to understand the differences observed between them.

2. Materials and methods

2.1. Bacterial strains

E. coli C600 K12 was used as recipient strain. *E. coli* TEM-1, *E. coli* TEM-2, *E. coli* TEM-3, *Klebsiella pneumoniae* SHV-2, *K. pneumoniae* SHV-3, and *K. pneumoniae* SHV-4 were used as donor strains. They were provided to us by Professor R. Labia (CNRS, Quimper, France). *E. coli* ATCC 25922 was used as the reference strain for determination of MICs.

2.2. Antibacterial agents

Amoxicillin (AMX), amoxicillin-clavulanic acid (AMC), ticarcillin (TIC), and ticarcillin-clavulanic acid (TCC) were from Smith Kline Beecham (Nanterre, France). Piperacillin (PIP) and piperacillin tazobactam (TZP) were from Wyeth Lederle (La Défense, France). Cefaclor (CEC) was from Lilly SA (Saint Cloud, France), and cefepime (FEP) was from Bristol-Myers-Squibb (Paris, France). Cefoxitin (FOX) was from Merck Sharp and Dohme-Chibret (Paris, France). Ceftazidime (CAZ) was from Glaxo Wellcome (Paris, France). Cefotaxime (CTX) was from Roussel and Diamant Hôpital (Paris, France). Ceftazidime (CAZ) was from Glaxo Wellcome (Paris, France). Gentamicin (GM), tobramycin (TM), and netilmicin (NET) were from Schering-Plough (Levallois-Perret, France). Amikacin (AN) was from Bristol-Myers-Squibb (Paris La Défense). Ciprofloxacin (CIP) was from Bayer Pharma (Putaux, France). Aztreonam (ATM) was from Sanofi Winthrop (Gentilly, France).

2.3. Susceptibility testing

MICs were determined by agar dilution technique on Muller-Hinton agar (Sanofi Diagnostics Pasteur, Marnes-la-Coquette, France). An inoculum of 10^4 CFU per spot was delivered with a multipoint inoculator (Well Repp2, Dynatech, Saint-Cloud, France) to a series of

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Table 1
MICs of various β -lactam agents for *E. coli* K12 and transconjugants (TEM and SHV)

Phenotype	MIC (μg ml ⁻¹) ^a													
	AMX	TIC	PIP	TZP	AMC	TCC	CEC	FOX	FEP	CAZ	CTX	CPO	ATM	IPM
SHV-2	> 512	> 512	32	1	4	16	64	2	0.25	0.5	0.5	0.25	0.25	0.06
SHV-3	> 512	> 512	128	8	8	16	256	4	0.25	1	2	0.5	1	0.03
SHV-4	> 512	> 512	128	4	8	16	512	4	1	64	2	0.5	128	0.125
TEM-3	> 512	> 512	256	2	8	32	128	2	0.5	8	4	1	2	0.125
TEM-2	> 512	> 512	256	16		64	16	1	< 0.06	< 0.06	< 0.25	< 0.06	< 0.06	0.03
TEM-1	> 512	> 512	128	8	8	64	16	4	< 0.06	0.125	< 0.03	< 0.06	< 0.06	0.125
<i>E. coli</i> K 12	4	2	1	1	4	2	4	1	0.06	0.03	0.03	0.03	0.03	0.06

^aAMX, amoxicillin; TIC, ticarcillin; PIP, piperacillin; TZP, piperacillin tazobactam; AMC, amoxicillin-clavulanic acid; TCC, ticarcillin-clavulanic acid; CEC, cefaclor; FOX, cefoxitin; FEP, cefepime; CAZ, ceftazidime; CTX, cefotaxime; CPO, cefpirome; ATM, aztreonam; IPM, imipenem.

agar plates which contained antibiotics in 2-fold dilutions. Incubation was for 16 h at 37°C. The MIC was determined as the lowest concentration of antibiotic at which no visible growth of three or fewer colonies was observed.

2.4. Transfer of resistance determinants

Cells of the donor strains and the recipient strains (10^9 CFU/ml strain) were mixed in Muller-Hinton broth (Sanofi Diagnostics Pasteur, Marnes-la-Coquette; France) and the mixture was incubated for 18 h at 35°C. Transconjugants *E. coli* K12 TEM-1, TEM-2, TEM-3, SHV-2, SHV-3, SHV-4 were selected on MacConkey agar supplemented with streptomycin (20 mg/l) and amoxicillin (100 mg/l). Streptomycin inhibited the growth of the donor strains but unaffected that of the recipient strains *E. coli* C600 K12, which was resistant to this antimicrobial agent. Amoxicillin allowed the growth of the donor strains but inhibited that of the recipient strains. The combination of these two antibiotics permitted the growth of the transconjugants [10]. Crude homogenates of β -lactamases were prepared as described previously [11].

2.5. Isoelectric focusing

Bacterial extracts were obtained by sonication (Sonicator: Sonifier 200, OSI, Paris, France) as described by Matthew et al. [2]. The pulsation time was 1 min with a sonication frequency of 30%/s. The sonicates were centrifuged at $10000\times g$ during 15 min at 4°C. The supernatant was frozen at -40°C for later use. Isoelectric focusing has been performed on the Phast System instrument (Pharmacia Biotech, St. Quentin, France). The isoelectric points of TEM-1, TEM-2, TEM-3, SHV-2, SHV-3, SHV-4 were respectively 5.4, 5.6, 6.3, 7.6, 7, 7.75. The gels (PhastGelND) used were characterised by pI in the ranges 3–9 and 5–8 (Pharmacia Biotech, St. Quentin, France) and were revealed by nitrocephin coloration (Oxoid Unipath Ltd., Basingstoke, UK).

2.6. Sample preparation, recording and treatment of ATR-FTIR spectra

The substrate used for ATR measurements was a ZnSe crystal ($50\times 10\times 1.5$ mm, from Specac, UK) with a refractive index of 2.4 and an incidence angle of 45° , yielding a total of six internal reflections at the sample. Bacterial colonies (18-h-old) were carefully harvested from the Muller-Hinton agar plates with a sterile polystyrene loop and homogeneously spread to cover the whole ATR crystal surface. Since a solid medium is used, this ensures that only bacteria are transferred to the IR substrate. It should be noted that the medium liquidizes only if it is kept at $45\text{--}50^\circ\text{C}$ for 30 min.

Spectra were recorded using a Bomem MB-100 (Vannier, Quebec) FTIR spectrometer equipped with a KBr beamsplitter and a DTGS detector. One-hundred interferograms were averaged per spectrum at a resolution of 4 cm^{-1} . For each strain, 10 spectra were recorded and the average spectrum was then normalised at the same integrated intensity in the spectral region of interest $1800\text{--}950\text{ cm}^{-1}$. Normalisation allows to reduce to the same number of bacteria, thus rendering spectra comparable. Reproducibility was in the range $\pm 2\%$.

2.7. Hierarchical cluster analysis

The dendrogram was constructed with the SPSS for Windows software using the average linkage between groups (UPGMA) method. This program considers each spectrum as a variable and each point of the spectrum as a parameter (here in the normalised region $1800\text{--}950$

cm^{-1}). It then calculates the shortest distance joining two given sets. Once these spectral distances were calculated, cluster analysis was performed in order to group the strains according to their IR-spectra. Two mean spectra, each calculated from 10 equivalent spectra were considered different if $r < 0.9997$, where r represents Pearson's coefficient and allows to measure the degree of similarity between two variables (IR spectra). In this study the intra-strain variability was found to be weak and less than the inter-strain one (data not shown).

2.8. Mathematical concept

Unresolved features in protein IR spectra make it difficult to gain more insight into detailed secondary structures. Increasing instrument resolution does not do away with this problem because the width of these contributing bands is generally bigger than adjacent peak-to-peak separation. So, mathematical concepts have to be applied to extract the hidden structural information. Fourier deconvolution, second derivative and curve-fitting are methods currently used [12].

3. Results and discussion

3.1. Susceptibility testing

The MICs of all strains used in this study are reported in Table 1. As it can be seen the six transconjugants of *E. coli* K12 (TEM-1, TEM-2, TEM-3, SHV-2, SHV-3, SHV-4) showed the same high resistance pattern to amoxicillin and ticarcillin but different susceptibility profiles to other β -lactams. For all six transconjugants, the acquisition of the plasmid in *E. coli* K12 did not confer resistance to aminoglycosides and quinolones. This excludes, respectively, a resistance due to nucleotidyltransferase and DNA gyrase. In view of this, the existence of only one mechanism due to a production of constitutively plasmid-mediated penicillinases can be assumed [13].

3.2. Classification

Cluster analysis, using average linkage between groups, has been applied to the mean normalised ATR-FTIR spectra of all seven strains and the dendrogram representation in Fig. 1,

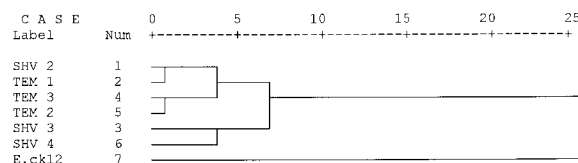


Fig. 1. Dendrogram resulting from a cluster analysis of the mean normalised ATR-FTIR spectra in region of interest ($1800\text{--}950\text{ cm}^{-1}$) for *E. coli* K12 and the transconjugants TEM and SHV. Two mean spectra, each calculated from 10 equivalent spectra, were considered different if $r < 0.9997$, where r represents Pearson's coefficient. The dashed line represents the rescaled distance cluster combine.

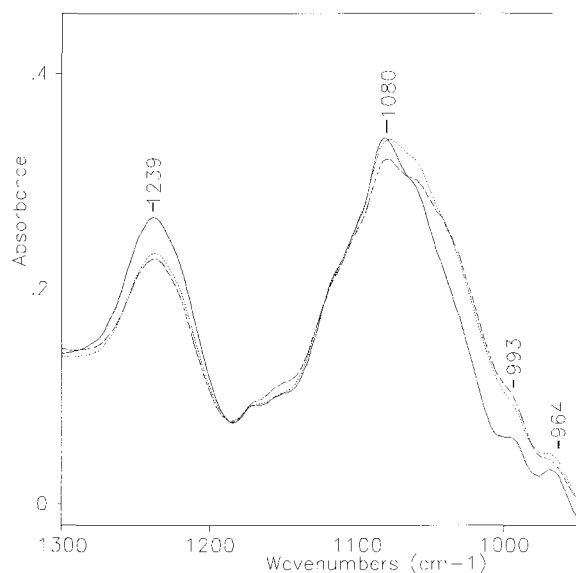


Fig. 2. ATR-FTIR mean normalised spectra showing the nucleic acids (1300–1200 cm^{-1}) and carbohydrate (1200–950 cm^{-1}) contours for the three strains: — *E. coli* K12 susceptible; ... *E. coli* K12 TEM-2; - - - *E. coli* K12 SHV-3. Conditions: 100 scans at 4 cm^{-1} resolution.

illustrates the discriminating power of this method. Differentiation between *E. coli* K12 susceptible strain and the transconjugants (TEM and SHV) can be clearly observed by com-

paring their distances. Indeed, the *E. coli* K12 strain was found to be located furthest away from the rest.

The SHV phenotypes were well characterised for SHV-2, but not for SHV-3 and SHV-4, since the last two display the same distance in the dendrogram. Characterisation of the TEM phenotypes showed similar distance in the dendrogram for TEM-2 and TEM-3 but different for TEM-1. Although these β -lactamases differ by specific point mutations that can be identified by DNA sequencing technique [13], our data based on phenetic classification, do not seem to detect such point mutations but rather give a global view of the consequences of the conjugation process (transfer frequency, experiment time, plasmids size, different promoters of TEM and SHV type enzymes, etc.). Thus, the differences observed within SHV and TEM transconjugants may be due to the expression, at the cellular level, of other genes constituting the plasmids.

To answer the question whether TEMs and SHVs can be discriminated, we have compared one member of each phenotype by considering the mean of 10 replicate spectra. Indeed, comparison between TEM-2 and SHV-3, on the one hand, with each other and, on the other hand, with *E. coli* K12 shows different distances in the dendrogram (Fig. 1).

3.3. Spectral analysis

The remarks made above concerning TEM-2 and SHV-3 can be explained by their different spectral profiles depicted in Fig. 2, specifically in the carbohydrate absorption region (1200–950 cm^{-1}). In addition to this, only absorption inten-

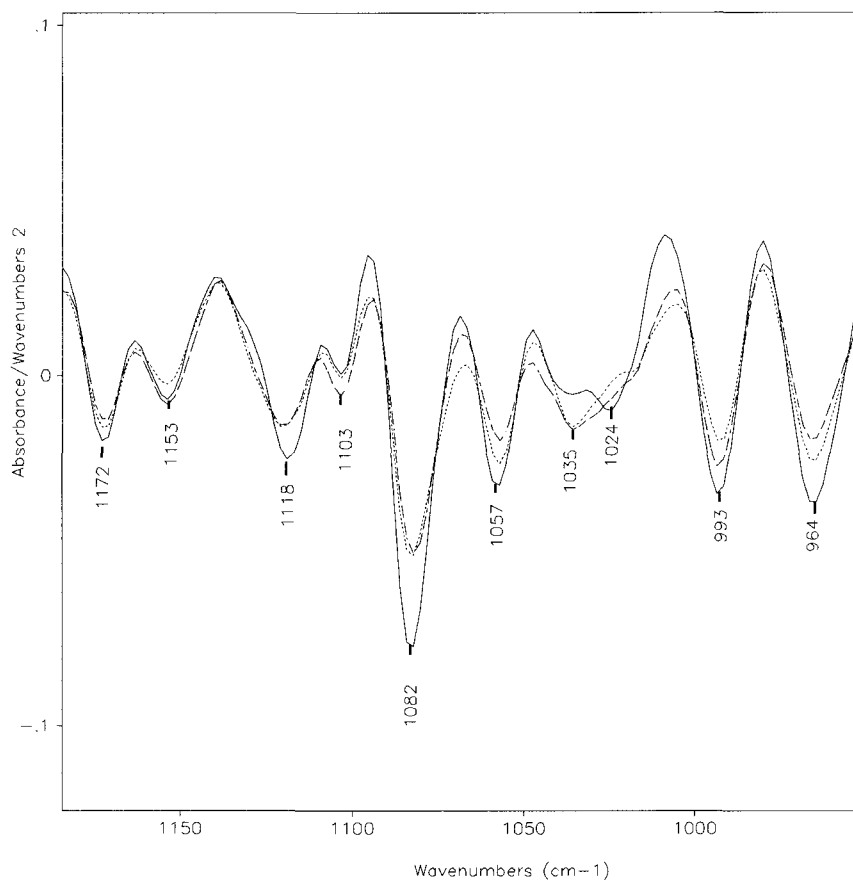


Fig. 3. Comparative second derivative ATR-FTIR spectra in the carbohydrate region 1200–950 cm^{-1} . — *E. coli* k12 susceptible; ... *E. coli* K12 TEM-2; - - - *E. coli* K12 SHV-3.

sity change was observed in the nucleic acids absorption region ($1300\text{--}1200\text{ cm}^{-1}$) [5] when the two strains were compared to the susceptible one.

By comparing the second derivative IR spectra of TEM-2, SHV-3 and *E. coli* K12, the following observations can be noted. For the transconjugants (TEM-2 and SHV-3) the absorption intensity was lower for the bands at 964 , 993 , 1057 , 1082 , 1118 , and 1172 cm^{-1} (Fig. 3). Another noticeable point is that for the transconjugants, the contour containing the bands at 1024 and 1035 cm^{-1} both from polysaccharides, and assigned respectively to CO, CC stretching motions and CO, CC stretching, COH bending motions [14] changes drastically. Indeed, the band located at 1024 cm^{-1} is clearly visible for the susceptible strain but very weak for TEM-2 and SHV-3 in the second derivative spectra. Conversely, the band at 1035 cm^{-1} is observed for TEM-2 and SHV-3 strains but only the onset is noticed for the susceptible strain in the derived spectra. This is because a 15-point second derivative was used.

However, the difference in spectral profile observed in the carbohydrate region for these three strains may be explained as follows. Both TEM- and SHV-type β -lactamases, produced in small quantities by *E. coli*, are cell-bound enzymes located in the periplasmic space. Their accumulation with other enzymes also present there suggests that they play some role in normal bacterial physiology [13]. A consequence of this co-existence is that the peptidoglycan synthesis, particularly the third phase, is influenced. This hypothesis seems more plausible to explain the differences in the ultrastructural and conformational status between the susceptible strain and the transconjugants bacterial cell wall polysaccharides. Moreover, amino-sugar of peptidoglycan and phospholipids (cell wall components) absorb strongly in the carbohydrate region ($1200\text{--}950\text{ cm}^{-1}$) [15]. However, the variation in the conformational status of these macromolecules can be observed at the spectral level [9].

A comparison of the contour area ($1300\text{--}1200\text{ cm}^{-1}$) assigned to asymmetric stretching of phosphate nucleic acids groups shows a relatively lower absorption intensity when TEM and SHV phenotypes are compared with the susceptible one. In fact, one expects in the conjugation process an increase in nucleic acid content since plasmids were added, but this is not observed. The cause-effect relationship may explain this last observation. Changes in the conformation status of both amino sugar of the peptidoglycan and phospholipids have direct repercussion in this region ($1300\text{--}1200\text{ cm}^{-1}$). This is felt by a lower absorption intensity in this region [15]. Besides, complicated coupled modes of vibration

that involves CH_2OH groups of polysaccharides are observed in this region [14].

In conclusion, our analysis of the IR spectra using cluster analysis based on overall similarity of bacterial strains, has clearly discriminated the transconjugants (SHV and TEM) from *E. coli* K12 susceptible strain. Although good results have been obtained for TEM-2 and SHV-3, there was no clear-cut in the distinction of SHVs from TEMs in general. The modifications in the spectral profile have been explained as a global effect of the conjugation process. This rapid and non-destructive method, requiring minimum sample-handling, may be of great benefit by helping to rapidly orientate investigations in bacterial resistance diagnostic.

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