Journal of Applied Microbiology 2000, **88**, 1001–1008

The effect of temperature and selective agents on the growth of *Yersinia enterocolitica* serotype O:3 in pure culture

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81/12/99: received 1 December 1999, revised 9 February 2000 and accepted 11 February 2000

C.M. LOGUE, J.J. SHERIDAN, D.A. MCDOWELL, I.S. BLAIR AND T. HEGARTY. 2000. This study examined the individual and combined effects of the selective agents normally present in *Yersinia*-selective agar (i.e. cefsulodin, irgasan and novobiocin) on the growth kinetics of plasmid-bearing (P+) and plasmid-cured (P–) *Yersinia enterocolitica* serotype O:3 at 25 and 37 °C. Growth studies were carried out in pure culture, and the data obtained were subjected to linear regression analysis to determine lag phase duration(s) and growth rates of the examined strains. In general, the presence of selective agents increased the duration of the lag phase at 37 °C, with longer lag phases noted in all cases in which two or more selective agents were present. Growth rates in CIN broth base (CIN NA) and CIN NA plus commercial supplement (SR 109) (CIN) were faster at 37 than 25 °C, but in some cultures of incomplete CIN NA broth with less than three supplements added, growth tended to be faster at 25 than 37 °C. Generally, plasmid-bearing strains grew slower than plasmid-cured strains in most media at 37 °C due to virulence plasmid expression retarding growth. In some instances at 37 °C, it was observed that the growth rates of both plasmid-bearing and plasmid-cured strains were comparable, indicating the influence of added selective agent/s negating any effects associated with virulence plasmid expression. The effects of selective agents, incubation temperature and virulence plasmid carriage on the growth kinetics of *Y. enterocolitica* are discussed.

INTRODUCTION

Yersinia enterocolitica is a significant foodborne pathogen causing gastroenteritis, and a range of severe sequalea in humans (Schiemann 1989). It is therefore important, to have an effective and rapid means for its isolation and or identification from suspect foods, environmental and clinical samples. Currently, the most common procedure involves selective plating on *Yersinia*-selective agar (CIN) (Schiemann 1979, 1982).

Schiemann (1980) investigated the growth of *Yersinia* in the presence of selective agents during studies to develop *Yersinia*-selective agar. Other workers have also examined the growth of *Yersinia* on a range of media (Fukushima and Gomyoda 1986; Toora *et al.* 1994). There is, however, little reliable data regarding the growth kinetics of *Yersinia* in such media, and the effect of the selective agents, i.e. cefsulodin, irgasan and novobiocin on this pathogen.

A number of studies (McDermott *et al.* 1993; Goverde *et al.* 1994) have shown that the choice of incubation temperature affects the growth kinetics of plasmid-bearing and plasmid-cured strains of *Escherichia coli* and *Y. enterocolitica*. In general, virulence plasmid expression in *Yersinia* occurs at temperatures > 30 °C, this expression is usually observed in phenotypic characteristics such as calcium dependency, autoagglutination and production of outer membrane proteins (Laird and Cavanaugh 1980; Cornelis *et al.* 1987; Bhaduri *et al.* 1990). Goverde *et al.* (1994) reported that virulence plasmid expression at temperatures greater than 30 °C retards the growth rate of plasmid-bearing *Y. enterocolitica* strains as plasmid replication places an additional metabolic burden on growing cells.

A previous investigation (Sheridan *et al.* 1998) reported that the growth rate of plasmid-bearing *Y. enterocolitica*

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strains were significantly faster at 37 °C than plasmid-cured strains in complete CIN medium. The present study was carried out to determine the effect of individual and combined selective agents on the growth rates of plasmid-bearing and plasmid-cured *Y. enterocolitica* and to determine the relationship between virulence plasmid expression, incubation temperature and selective agents.

MATERIALS AND METHODS

Yersinia enterocolitica culture

Y. enterocolitica GER, serotype O:3 P+ and P– cultures were supplied by Dr S. Bhaduri (USDA, ARS, ERRC, Philadelphia, PA, USA). The P+ cultures were stored frozen on beads (Protect, Technical Consultant Services Ltd, Heywood, UK) and the P– cultures maintained on tryptone soya agar slopes and plates (TSA, CM 131, Oxoid, Unipath Ltd, Basingstoke, UK) as described previously (Sheridan *et al.* 1998).

Inoculum preparation and enumeration

The P+ and P– inocula were prepared and enumerated as described previously (Sheridan *et al.* 1998). Briefly, cultures of P+ and P– strains were inoculated into 9·0 ml volumes of brain heart infusion broth (BHI, CM 225, Oxoid) and incubated overnight at 25 °C. The cultures were serially diluted in sterile, filtered maximum recovery diluent (MRD, CM 733, Oxoid), and the numbers of *Y. enterocolitica* per millilitre of suspension determined using a membrane filtration epifluorescent staining technique (Walls *et al.* 1989). The enumerated suspension was diluted in MRD to give a working inocula and the cell concentrations were confirmed on *Yersinia*-selective agar (CIN, CM 653, SR 109, Oxoid).

Broth preparation

Yersinia-selective broth base (CIN NA) was prepared from first principles according to the Oxoid Manual (6th edition 1990) (Sheridan *et al.* 1998).

Selective supplements

Individual solutions of the selective agents of *Yersinia*-selective supplement were prepared on the day of use.

Cefsulodin (sodium salt, Sigma-Aldrich Co. Ltd, Poole, UK), 7·5 mg was dissolved in 2 ml of sterile distilled water.

2, 2, 4′-trichloro-2′ hydroxy diphenyl ether (Irgasan DP 300) (Ciba Geigy Dyestuffs and Chemicals, Manchester, UK), 2·0 mg was dissolved in 2·0 ml of ethanol (Merck Chemicals Ltd, Darmstadt, Germany).

Novobiocin (monosodium salt, Merck), 1·25 mg was dissolved in 2·0 ml of sterile distilled water.

Yersinia-selective supplement (CIN) (SR 109, Oxoid) was prepared by the addition of 2 ml of a 1 : 1 ethanol and sterile distilled water mixture to one vial of supplement.

Culture studies

Two-hundred and twenty-five millilitre volumes of CIN NA broth base were temperature equilibrated at 25 or 37 ± 1 °C, in a thermostatically controlled water-bath (Lauda, Königshofen, Germany) for approximately 1 h before use. The broth was transferred to a sterile 250-ml nalgene flask (Nalgene, Nunc Int., Rochester, NY, USA) fitted with a sterile temperature probe and catheter needle (Becton and Dickinson, Dublin, Ireland) attached. Aliquots (0·9 ml) of the individual selective agents (i.e. cefsulodin, irgasan, novobiocin), or combinations of these agents, or the commercial CIN supplement (SR 109) were mixed into the broth base.

Pure culture systems were prepared by adding a 10·0-ml inocula of *Y. enterocolitica* P+ or P– strains containing approximately 23 500 cfu ml^{-1} to the supplemented CIN NA broths to give initial concentrations of approximately 1000 cfu ml^{-1}. The mixtures were shaken thoroughly to disperse the inocula within the broths. All culture systems were incubated in water baths at 25 or 37 ± 1 °C for 9 h. Every hour, a 5·0-ml sample of culture was withdrawn from each culture using a sterile syringe (Becton and Dickinson) and examined as described below. Each experiment was repeated on three occasions.

Growth studies were carried out for the two *Y. enterocolitica* strains (P+ and P–) in CIN NA broth, in CIN NA broth supplemented with single selective agents (i.e. cefsulodin, irgasan, novobiocin), and with combinations of two selective agents, as well as in CIN NA broth containing CIN supplement.

Generation of growth curves

Volumes (1·0 ml) of the culture were serially diluted in MRD and duplicate aliquots of each dilution series were plated out on *Yersinia*-selective agar (CIN) using a spiral plater (Model D, Spiral Plating Systems, Cincinnati, OH, USA). Plates were incubated at 37 °C for 24 h and the numbers of *Y. enterocolitica* (cfu ml^{-1}) colonies on each plate were enumerated using image analysis (Seescan Imaging Systems, Cambridge, UK). Counts obtained were plotted against time to produce growth curves.

Statistical analysis

All growth rate data from these studies was analysed using linear regression analysis (Systat, Evanston, IL, USA). The lag phase durations and growth rates of each *Y. enterocolitica* strain were determined for each temperature/selective agent regime according to Broughall *et al.* (1983). Briefly, all data were included from the points at which the cell concentration had increased to 150% of the inoculated concentration, to the point where the population density ceased to increase. The specific lag times and growth rates of *Yersinia* in the presence of the different supplements were determined as previously described (Duffy *et al.* 1994).

Statistical analysis was carried out from the design of the experiment which consisted of randomized blocks of data containing two strains (P+ and P–), eight enrichment broths (CIN NA, CIN NA + cefsulodin, CIN NA + irgasan, CIN NA + novobiocin, CIN NA + cefsulodin and irgasan, CIN NA + cefsulodin and novobiocin, CIN NA + irgasan and novobiocin, CIN NA + CIN supplement), and two incubation temperatures (25, 37 °C) in factorial combination. This design was used for both lag phase and growth rate data analysis. Analysis of variance (ANOVA) was carried out on lag phase and growth rate data using Genstat 5 (Rothamsted Experimental Station, Harpenden, UK). The standard error of difference was determined from the ANOVA. Results presented are a mean of three replicates. *t*-tests were carried out to compare the lag phase durations of the strains in different supplemented media, a similar procedure was used for growth rate data. The significance of the differences between the means was determined using the least significance approach (Steel and Torrie 1980).

RESULTS

Table 1 shows the effect of single or combinations of selective agents in CIN NA broth on the lag phase duration of *Y. enterocolitica* P+ and P– strains incubated at 37 and 25 °C. In general, lag phases were longer at 37 than 25 °C. An exception to this pattern was noted for *Yersinia* cultures in CIN NA broth at 25 °C where the lag phase could not be determined (Sheridan *et al.* 1998). It was also noted that the lag phases were generally longer in the presence of the selective agents for both temperatures examined.

When the influence of selective agents on the lag phase durations of P+ and P– strains were compared at 37 °C; significantly longer lag phases ($P < 0{\cdot}01$) were noted for P+ strains than P– strains in CIN NA broth supplemented with cefsulodin and novobiocin. While, P– strains had a significantly longer ($P < 0{\cdot}05$) lag phase in the presence of irgasan or full CIN supplement.

Table 1 The effect of selective agents on the lag phase durations (h) of *Yersinia enterocolitica*

	Incubation temperature (°C)			
	37		25	
	P+	P –	P+	P –
Broth				
CIN NA	0·41	0·51	NC	NC
CIN NA + cefsulodin	0·45	0·57	0·94	0·69
CIN NA + irgasan	1·04	1·65	0·47	1·09
CIN NA + novobiocin	0·96	0·85	0·70	0·62
CIN NA + Cef + Irg	0·81	0·78	0·55	0·74
CIN NA + Cef + Nov	1·34	0·64	0·12	0·28
CIN NA + Irg + Nov	1·75	1·40	0·39	0·50
CIN NA + CIN supplement	0·88	1·58	0·92	0·69

NC – not calculated, SED – 0·24, CIN NA – *Yersinia*-selective broth base, degrees of freedom = 58.

At 25 °C, the lag phase duration of P+ and P– strains were generally similar. It was noted, however, that the lag phase of P– strains was significantly longer ($P < 0{\cdot}05$) than P+ where irgasan was present.

The overall duration of the lag phase of *Y. enterocolitica* P+ and P– strains was influenced by both the selective agents present and the incubation temperature. Longest lag phases for P+ strains were noted in CIN NA broth supplemented with irgasan and novobiocin at 37 °C, and for P– strains in the presence of irgasan. At 25 °C, P– strains had the longest phase in the presence of cefsulodin or CIN supplement, while P– strains had the longest duration where irgasan was present.

When the lag phase durations were compared between the incubation temperatures, they were generally longer in cultures incubated at 37 °C. P+ strains had significantly longer ($P < 0{\cdot}05$) lag phase duration at 37 °C where irgasan, or combinations of selective agents, i.e. cefsulodin and novobiocin, irgasan and novobiocin, were present. In contrast, the lag phase duration for P+ strains was significantly longer ($P < 0{\cdot}05$) at 25 than 37 °C in the presence of cefsulodin.

With P– strains, the lag phases were significantly longer at 37 than 25 °C in CIN NA broth containing irgasan ($P < 0{\cdot}05$) and with combinations of irgasan and novobiocin and full CIN supplement ($P < 0{\cdot}001$).

Examination of the growth rate data revealed a linear relationship between bacterial numbers and time during the growth phase of *Y. enterocolitica* P+ and P– cells. High correlation coefficients (r^2 values) were observed under most growth conditions with r^2 values ranging from 0·71 to

Table 2 The effect of selective agents on the growth rates (cfu $ml^{-1} h^{-1}$) of *Y. enterocolitica*

	Incubation temperature (°C)			
	37		25	
	P+	P –	P+	P –
Broth type				
CIN NA	0·33	0·37	0·16	0·12
CIN NA + cefsulodin	0·29	0·43	0·34	0·34
CIN NA + irgasan	0·38	0·38	0·34	0·32
CIN NA + novobiocin	0·41	0·40	0·32	0·34
CIN NA + Cef + Irg	0·14	0·28	0·31	0·33
CIN NA + Cef + Nov	0·25	0·34	0·32	0·28
CIN NA + Irg + Nov	0·12	0·23	0·34	0·30
CIN NA + CIN supplement	0·40	0·28	0·29	0·28

SED = 0·03, degrees of freedom = 63.

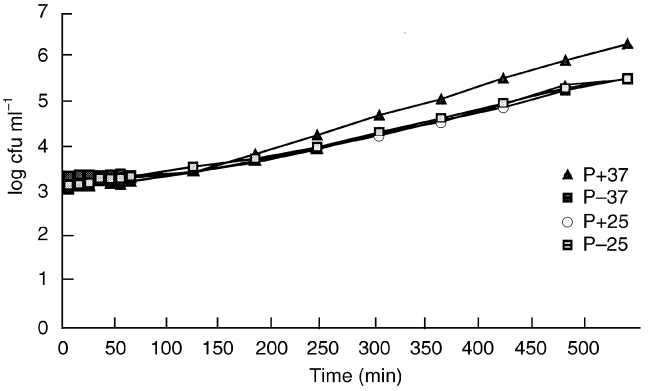

Fig. 2 The growth of *Yersinia* in CIN NA broth + CIN supplement

0·99. The low r^2 value ($r^2 = 0·71$), associated with the growth of P+ strains in CIN NA broth supplemented with cefsulodin and irgasan, was attributed to a high RSD value, indicating a large variation between replicates.

Table 2 shows the effect of selective agents on the growth rate of *Y. enterocolitica* P+ and P– strains. Generally, the growth rates of *Yersinia* were faster at 37 than 25 °C (Fig. 1). When the growth rates of P+ and P– strains were compared at 37 °C, the plasmid-cured strain (P–) grew significantly faster ($P < 0·01$) than P+ strain in most media. Exceptions were noted in CIN NA + CIN supplement where the opposite case occurred with significantly faster ($P < 0·001$) growth rates noted for P+ strains (Fig. 2) and in the presence of irgasan and novobiocin where no differences were observed. At 25 °C, no differences were observed in the growth rates of P+ or P– strains.

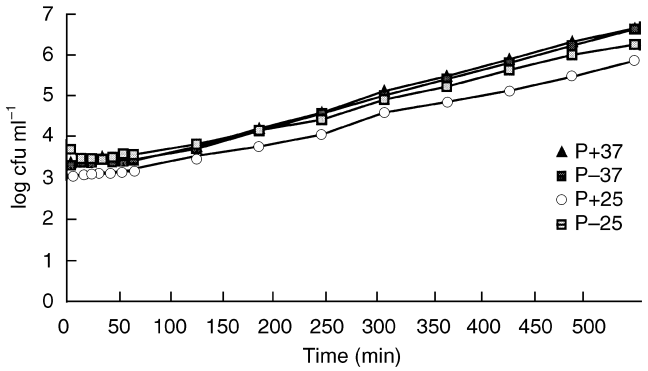

Fig. 1 The growth of *Yersinia* in CIN NA broth + novobiocin

The overall growth rate of the *Yersinia* strains was influenced by both the selective agents present and the incubation temperature. P+ strains grew fastest in CIN NA broth at 37 °C supplemented with novobiocin or CIN supplement, while P– strains grew fastest in the presence of single selective agents, i.e. cefsulodin, irgasan or novobiocin. At 25 °C, the growth rates of P+ and P– strains were generally similar although slower growth rates were noted in CIN NA broth for both strains.

When the growth rates of P+ and P– strains were compared between temperatures, P+ strains grew significantly faster ($P < 0·01$) at 37 than 25 °C in CIN NA broth and in the presence of novobiocin and full CIN supplement. Significantly faster growth rates were noted at 25 than at 37 °C where combinations of supplements, i.e. cefsulodin and irgasan, irgasan and novobiocin, and cefsulodin and novobiocin ($P < 0·05$) were present.

With the plasmid-cured strain (P–) growth was significantly faster ($P < 0·05$) at 37 °C in the presence of most supplements, except in the presence of irgasan and novobiocin where growth was faster at 25 °C ($P < 0·05$) or with full CIN supplement where no differences were observed.

Overall, selective agents extended the lag phase duration of *Y. enterocolitica* P+ and P– strains at 37 °C, when one or more combinations of selective supplements were present. When the growth rate data were examined, there was an association between strain type and incubation temperature. Plasmid-cured strains generally grew faster than plasmid-bearing strains at 37 °C in most media indicating virulence plasmid expression at the higher temperature retarded P+ strains. One exception was observed, however, where P+ strains grew significantly faster than P– in the

presence of CIN supplement, indicating a possible association between selective agents, incubation temperature and virulence plasmid expression. In contrast, the growth rates of the plasmid-cured strain appeared to be retarded at 37 °C to rates similar to those observed at 25 °C.

DISCUSSION

This study aimed to determine the individual and combined effects of selective agents on the growth kinetics of plasmid-bearing (P+) and plasmid-cured (P–) *Y. enterocolitica* cells and to elucidate the effects of strain growth kinetics, strain type, selective agent(s) and incubation temperature.

Sheridan *et al.* (1998) reported that at 37 °C, the lag phase of *Y. enterocolitica* in CIN NA broth plus selective agents was longer than the lag phase observed in CIN NA broth. A similar pattern was observed in a study of *Listeria monocytogenes* (Sheridan *et al.* 1994), which also reported an extension of the lag phase duration in the presence of a selective supplement. The current study confirmed these observations in relation to the effects at 37 °C, however, no extended lag phase duration was observed in supplemented CIN NA broth at 25 °C. The shortest lag phase occurred at 25 °C in the presence of cefsulodin and novobiocin. Virulence plasmid expression did not appear to influence the lag phase duration. It is difficult to interpret these effects, but temperature appears to play an important part.

The presence or absence of a virulence plasmid had an effect on growth rate, i.e. plasmid-bearing *Y. enterocolitica* strains grew slower than plasmid-cured cells at 37 °C. This observation is in agreement with previously reported results (Sheridan *et al.* 1998), and may be associated with the additional metabolic burden involved in the expression of the virulence plasmid (McDermott *et al.* 1993; Goverde *et al.* 1994).

This study observed that growth was slow in CIN NA broth at 25 °C. This may be due to the inhibitory nature of the broth base, as previously reported (Fukushima and Gomyoda 1986; Sheridan *et al.* 1998). A previous study by Sheridan *et al.* (1998) showed that the inhibition could be reduced by increasing the incubation temperature to 37 °C or by inclusion of the two antibiotics and the surfactant, irgasan, in the medium at 25 °C. The present study confirmed these observations, and also found that the inhibition could be reduced by the addition of one or more of the selective agents (cefsulodin, irgasan or novobiocin).

Sheridan *et al.* (1998) suggested a number of possible reasons for the absence of inhibition under such conditions. These included (a) the production of substances by the cells that complex the inhibitory agent or limit its activity or (b) as a result of changes in cell structure that prevent or reduce the access of inhibitors into the cell. Both these processes could be operational in cultures grown at 37 °C. When plasmid-bearing *Yersinia* cells are grown at 37 °C, they produce significant amounts of *Yersinia,* outer membrane proteins (YOPs) which could bind the inhibitor (Bhaduri 1990; Cornelis 1992). It is well known that most cells adapt the composition and characteristics of the cell membrane in response to environmental changes, specifically producing a more rigid cell membrane at 37 °C than at 25 °C, these changes can result in reductions in membrane permeability (Tsuchiya *et al.* 1987; Nagamachi *et al.* 1991). This effect is associated with the levels of saturated and unsaturated fatty acids present in the outer membranes. At higher temperatures, the saturated fatty acid content increases and unsaturated fatty acids decrease (Nagamachi *et al.* 1991). Membranes with increased saturated fatty acids are less permeable than membranes with high unsaturated fatty acid contents (Cornelis *et al.* 1987; Tsuchiya *et al.* 1987; Nagamachi *et al.* 1991; Goverde *et al.* 1994; Bodnaruk and Golden 1996). As well as these changes in lipid content, incubation at higher temperatures causes a number of qualitative and quantitative changes in membrane-associated proteins. Outer membrane porins are no longer synthesized (Cornelis *et al.* 1987; Rhode *et al.* 1994). These porins have an important role in the transport of a range of substances, including antibiotics across the outer cell membrane (Brown and Williams 1985; Brozostek and Hrebenda 1988). Thus, at the higher temperatures, reductions in porin concentrations would reduce the access of selective agents to the cell interior, limiting the inhibitory impact of such agents on cellular growth.

The calcium content of the growth medium may also play a role in modulating the effects observed in this study. At 37 °C, plasmid-bearing (P+) *Y. enterocolitica* cells require specific concentrations of Ca^{2+} in growth media to support normal growth. In the absence of adequate calcium, cells can cease growing and produce a number of YOPs which are released into the growth medium. YOPs are encoded by the virulence plasmid and their expression is temperature and calcium regulated (Cornelis *et al.* 1987; Bhaduri 1990; Michiels *et al.* 1990; Li *et al.* 1998). Sheridan *et al.* (1998) reported that the calcium level in CIN NA medium was 325 μmol l^{-1} Li *et al.* (1998) recently reported that Ca^{2+} levels above 745 μmol l^{-1} were required to preserve virulence plasmid and prevent YOP production, it is therefore likely that the calcium concentration in the CIN NA broth would induce a low calcium response.

At 25 °C, two major effects were observed for plasmid-cured (P–) strains, similar to cultures incubated at 37 °C, i.e. growth was not prevented by any of the antibacterial agents alone, or in combination with irgasan, growth inhibition in the CIN NA medium did not occur. As noted previously, cells incubated at 25 °C increase their permeability by altering the outer membrane. This is facilitated

by changes in the levels of unsaturated fatty acids, and an increased synthesis of porins. Despite this, neither of the antibiotics or irgasan used in the present study resulted in inhibition of cell. This is in line with previous reports on the effects of novobiocin (Raevuori *et al.* 1978; Schiemann 1980; Toora *et al.* 1994), cefsulodin (Toora *et al.* 1994) a combination of irgasan and novobiocin (Toora *et al.* 1994) or all three substances together (Toora *et al.* 1994). In their work at 25 °C Toora *et al.* (1994) showed that all three substances added to the medium together were more inhibitory than single or double antibiotics, which was generally the case in the present study.

Given the increased membrane permeability of cells and increased porin carrier availability at 25 °C, it would be reasonable to assume that the selective agents should have greater impact in reducing growth rates at this temperature, however, this was not the case. The general lack of inhibition by antimicrobials and the negation of inhibition observed in unsupplemented media by the creation of a more inhibitory environment are difficult to explain. They may be the net outcome of a number of conditions that apply to *Yersinia* cells incubated at 25 °C. While porins are present at this temperature, substances such as cefsulodin, novobiocin and irgasan are hydrophobic which results in their having a low cell permeability (Nikaido and Nakae 1979; Meincke *et al.* 1980; Nikaido *et al.* 1983).

The O-antigen glycoside side chains of *Yersinia* are longer and project further from the surface of the organism in strains cultured at 25 °C, compared with similar strains incubated at 37 °C (Skurnik and Toivanen 1993; Goverde *et al.* 1994). These extended chains may present a more significant steric barrier limiting the entry of substances into the cell, alternatively, such external chains may interact with selective agents preventing them from continuing through the cell membrane to their site of action within the cell (Skurnik and Toivanen 1993; Goverde *et al.* 1994). Steric protection of *Yersinia enterocolitica* cells by cell surface components has been reported by Martinez (1983) who noted that cells cultured at 25 °C were able to withstand the killing action of serum compliment, compared with similar cells cultured at 37 °C. Such cell surface/capsule adaptations could limit or prevent the entry of inhibitory substances including antibiotics and surfactants into *Yersinia* cells.

At 37 °C, the addition of single antibiotics generally had no effect on growth rate of *Y. enterocolitica*. Overall growth at 37 °C was faster than at 25 °C, indicating an increased susceptibility of *Yersinia* to antibiotics at lower temperatures (Kouwatli *et al.* 1979). In keeping with the data at 25 °C, virulence plasmid expression did not have any effect on growth rates, except in the presence of cefsulodin, where plasmid expression significantly decreased the growth rate. According to Robins-Browne *et al.* (1986) in the presence of the virulence plasmid the minimum inhibitory concentrations of a number of antibiotics to *Yersinia enterocolitica*, including novobiocin, was significantly less at 37 °C in plasmid-bearing cells. A similar result has also been reported by Jimenez-Valera *et al.* (1989) for other antibiotics. In studies of *E. coli* it was shown that the presence of a Col V plasmid increased the sensitivity of the organism to novobiocin and rifampicin (Davies *et al.* 1986).

In this study the most notable effects observed were when each of the antibiotics and the surfactant were combined together in the growth medium at 37 °C. In the presence of the virulence plasmid, all combinations were significantly more inhibitory on growth and the data show that irgasan greatly enhanced the inhibitory effect of both antibiotics.

It has been shown that drugs may act synergistically with surfactants to increase their activity on cells. This may result in an increased transfer of the drug across the outer membrane or in an increase in the binding of the drug to the receptor sites (Attwood and Florence 1985). Irgasan acts as a site-directed inhibitor of enoyl-acyl carrier protein reductase by mimicking its natural substrate, thus inhibiting fatty acid synthesis and, consequently, lipid formation (Levy *et al.* 1999). Such inhibition will dramatically affect the formation of the outer membrane and increase antibiotic transfer across the barrier. As well as these effects, surfactants may also bind proteins in solution and the possible significance of these interactions could explain some the inhibitions observed in this study.

Although the synergistic activity between irgasan and the antibiotics is a possible method of activity of surfactants and antibiotics inhibiting cells, it should be noted that this type of inhibition was only observed for plasmid-bearing cells. This would support the view that some form of protein/surfactant interaction resulted in an increased antibiotic activity. A possible mechanism for this may be as follows.

Irgasan is hydrophobic and lipophilic in nature, and sufficiently soluble to be absorbed into the hydrophobic areas of cell walls and membranes. According to Meincke *et al.* (1980) irgasan is adsorbed to the outer cell membrane which behaves as a barrier to its entry into the cytoplasmic membrane, which is its site of action. It has been suggested (Meincke *et al.* 1980) that irgasan is adsorbed onto the outer cell membrane which prevents penetration to and damage of the inner cytoplasmic membrane. Such irgasan/membrane complexes could interact with large amounts of outer membrane proteins present in outer membranes of plasmid-bearing *Yersinia* growing at 37 °C, disrupting protein membrane structures, leading to increased membrane permeability (Attwood and Florence 1985). Cell membranes damaged in this way could be more easily penetrated by the antibiotics, which would mean that such

antibiotics would more easily gain access to and bring about damage of the cytoplasmic membrane. Alternatively, the important interactions between irgasan and antibiotics may occur after they have separately penetrated the cell. Irgasan as the surfactant may increase the avidity of the antibiotics to their sites of action within the cell (Attwood and Florence 1985).

This study noted that under some circumstances the presence of antibiotic increased the growth rates of cells. Thus, growth rates in the presence of each antibiotic are among the highest noted within the study. As antibiotics are normally viewed as suppressing bacterial growth, these observations would appear, on first analysis, to be unusual. However, there are previous reports of the enhancement of growth in the presence of antibiotics. Novobiocin has been previously reported to cause increases in the growth rates of cells (Drilica and Rouviere-Yaniv 1993) and this has been associated with the enhancement of enzyme/DNA gyrase activities in growing cells (Drilica and Rouviere-Yaniv 1993; Sheridan *et al*. 1998).

Rapid growth was also observed in other circumstances where inhibition may have been predicted. Thus, in complete CIN, i.e. CIN NA broth with full selective supplement, the presence of both antibiotics and the surfactant, might have been expected to be considerable if not maximal. This phenomenon could not be explained satisfactorily but emphasizes that complex interactions occur between antibiotics and surfactants and the major influence that the virulence plasmid expression can have on the growth of the organism is at 37 °C.

This study raises questions in relation to the development of media for the growth or isolation of pathogens from foods. Despite recent advances, the ability to rapidly and comprehensively recover and/or differentiate organisms in liquid or agar media still remains the cornerstone of many areas of microbiology (Bloomfield *et al*. 1998). The study raises questions in relation to the development of media for the growth or isolation of *Y. enterocolitica* or other significant pathogens from foods. Depending on the net effect of the interactions of physiological and genetic status of the strains, the synergistic and antagonistic interactions between media components and other aspects of cultural conditions growth may be significantly enhanced or reduced. Of greater concern are the observations in this study which demonstrate that the construction of media is not simply a process of identifying contaminants that need to be suppressed and the selection of agents which are more active against the contaminant than the target organism, and combining these agents in a cost-effective format. More important is examining the effect of adding substances together in media without determining their combined effects. The results of selecting multiple antibiotics may be inhibitory, while if present alone they will have no effect. In this study it was shown that the presence of surfactants may increase or decrease the effects of other inhibitory agents, in unpredictable ways. Such effects are rarely taken into account on the development or application of selective media. Yet the availability of media for the rapid, efficient selection and cultivation of bacterial species of interest remains central to most microbiological research and practice. In conclusion, further detailed research into the phenotypic and genetic status of bacteria and their physiological interactions with current and potential components of selective media is important in the development of more efficient methods for the recovery, isolation and analysis of many important bacterial species.

ACKNOWLEDGEMENT

The authors gratefully acknowledge the financial assistance of the European Union.

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