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Efficiency of Different Chemical and Physical Agents for Curing of Plasmid/s from Salt Tolerant Bacterial Strains

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Abstract: Curing efficiency was higher with different TMP concentrations in combination with alkaline pH (9) at 37 as well as 42°C. Both strains could tolerate up to 3.5 M NaCl in L agar but in L broth RCa-1 could deter 2 M and Ca-2 upto 2.5 M NaCl. Curing was accomplished with loss of salt tolerance property. Salt tolerance among the cured derivatives ranged from 0.1 M (majority) to 0.5 M (few) in L broth. Generally, Na⁺ ion uptake was more in the cured derivatives as compared to their parental strains when grown in 0.1 M Neel supplemented broth under optimum temperature and pH. A comparison of cell wall composition of parental strains and their cured derivatives exhibited more peptidoglycan (PG), diaminopimelic acid (DAP) and teichoic acid contents in the plasmid free isolates.

Key words: Chemical, physical, plasmid/s, salt, tolerant, bacterial, strains

Introduction

Two of the biggest concerns to the farm viability and environment in Pakistan are problems of salinity/sodicity and water logging. One way of characterizing plasmid is to cure plasmids from the host strain and study plasmid associated characters. Curing refers to the complete loss of plasmid from bacterial strains and loss of the property from the bacterial host is evidence of plasmid controlled attributes. Since no universally affective curing agent has been identified, curing experiments are generally conducted on trial and error basis, both with respect to the choice of curing agents and culturing conditions used. Both physical and chemical agents are used for this purpose (Caro *et al.*, 1984; Stanisich, 1988; Gerhardt *et al.*, 1994; Yasmin and Hasnain, 1996; Muzzamal, 1998). Line of action of these agents is either interference with DNA replication or affecting a particular organelle or enzyme of the bacterial cell (Stanisich, 1988). While some compounds may cause curing in a rather non specific way. Among various chemical and physical agents reported for successful isolation of plasmid free cells include intercalating dye like acridine dye, ethidium bromide, sodium dodecyl sulfate (SDS) (Stanisich, 1988) and antibiotics like trimethoprim (TMP), novobiocin (Hooper *et al.*, 1984; Diver and Wise, 1986), high temperature (Stanisich, 1988; Lebrun *et al.*, 1992) and pH (Saint and Venables, 1990; Lakshmi and Thomas, 1996; Qazi and Hasnain, 1997).

In spite of the fact that curing has been reported successfully in the bacterial strains with these agents but curing conditions and curing agents are specific for particular plasmid. The present work deals with the optimization of curing conditions for removal of plasmid from two halotolerant bacteria (a pseudomonad, RCa-1 and a member of Vibrionaceae, Ca-2). Curing efficiency of different combinations of TMP concentrations, temperatures and pH were investigated. Effects of curing on cell and colony morphology, some cell wall constituents and Na uptake in the two bacteria are also being reported.

Materials and Methods

Different chemical (IMP-125, 150, 175 µg ml⁻¹ conc.) and physical (temperature-28, 37 and 42°C; pH-6, 7, 9) agents, in various combinations, were used for curing of plasmids in two salt tolerant bacterial strains (RCa-1 and Ca-2). The strains were isolated from rhizoplane (RCa-1) and histoplane (Ca-2) of *Chenopodium album* growing along Riwind road near Lahore.

The salt tolerant bacterial strains RCa-1 and Ca-2 belonging to Pseudomonadaceae and Vibrionaceae, respectively, were used for

curing experiments. These strains were isolated from histoplane (Ca-2) and rhizoplane (RCa-1) of *Chenopodium album* and could tolerate 2.0-2.5 M NaCl in nutrient agar and 1 min liquid medium. The optimal temperature of RCa-1 was 28°C while Ca-2 gave maximum growth at 37°C while optimum pH of these strains was 9. Plasmids pSH1403 and pSH1402 reside in RCa-1 and RCa-2, respectively (Yasmin and Hasnain, 1998). Normally these strains were grown in L broth and L agar (Gerhardt *et al.*, 1994) supplemented with 1 M NaCl at 37°C.

From overnight cultures at 37°C inoculum was given in L broth adjusted at different pHs (6, 7, 9) containing different concentrations of trimethoprim (TMP-125, 150, 175 µg ml⁻¹) and incubated at 28°, 37°, 42°C. Cultures were subcultured in prewarmed L broth (with specific pH) containing the respective TMP concentrations after every 24 hours of incubation at respective temperatures upto three subcultures. At the time of subculturing samples were drawn and dilutions were plated on L agar plates with respective pHs (6, 7, 9) and incubated at the specific temperatures. Salt sensitive colonies were screened by replica plating on NaCl (1 M) and L agar plates with respective pHs (6, 7 and 9) and incubating at specific temperature (28, 37 and 42°C). Removal of plasmid was authenticated by the gel electrophoresis of total cell lysate (Thomas, 1984) of salt sensitive colonies.

Na uptake by the parental strains and their cured derivatives grown for 24 hours at 37°C in 0.1 M NaCl supplemented L broth was determined on flame photometer (Furman, 1975).

Cell wall of the two parental strains and their cured derivatives were purified ensuing by Leduc *et al.* (1989) and Hancock (1994). Method of Work (1957) was followed for DAP (diaminopimelic acid) estimation and those of Leduc *et al.* (1989) and Hancock (1994) for PG (peptidoglycan estimated as hexosamines i.e. glucosamines) and TA (teichoic acid), respectively.

Results

Before curing experiments M.I.C. (minimum inhibitory concentration) and LD₅₀ of trimethoprim was determined for the two strains. M.I.C. and LD₅₀ for two salt tolerant strains were 80 and 150 µg ml⁻¹ TMP, respectively. LD₅₀ and two other TMP concentrations (125, 175 µg ml⁻¹) were used for curing experiments.

For plasmid curing, salt tolerant strains were grown in different concentrations of TMP (125, 150, 175 µg ml⁻¹), at three different pHs (6, 7, 9) and three temperatures (28, 37 and 42°C) upto three subcultures. Hundred colonies (or less in those cases where number of single colonies obtained were less) from each combination at

every subculture were screened for salt sensitivity by replica plating and removal of plasmid from salt sensitive colonies was ascertained by gel electrophoresis of total cell lysate. Response of the two salt tolerant strains RCa-1 and Ca-2 harbouring plasmids pSH1403 and pSH1402, respectively, varied with different combinations of curing conditions.

No salt sensitive colony was obtained at acidic pH for both of strains (except Ca-2, II subculture, 175 $\mu\text{g ml}^{-1}$ TMP). The salt sensitive colonies were obtained in first subculture at alkaline pH while in neutral medium salt resistance was lost in subsequent subcultures. Effective TMP conc. was different in all the three cases. However for RCa-1 combination of high temperature and alkaline pH with different concentrations of TMP (125, 150, 175 $\mu\text{g ml}^{-1}$) yielded salt sensitive colonies at all subcultures (except RCa-1 at 175 $\mu\text{g ml}^{-1}$ TMP, II subculture and 150 $\mu\text{g ml}^{-1}$ TMP, III subculture). Whereas for Ca-2 salt sensitive colonies were obtained at neutral and alkaline pH with varying temperatures. Generally the percentage of salt sensitive colonies decreased in subsequent cultures. In RCa-1 all the three temperatures at alkaline pH proved effective to yield salt sensitive colonies at subculture I, but the proportion of salt sensitive colonies was higher at 28° and 42°C. At 37°C salt sensitive colonies were obtained at 150 and 175 $\mu\text{g ml}^{-1}$ TMP. While in Ca-2 at alkaline pH salt sensitive colonies were observed at 28° and 37°C (I subculture) but not at 42°C. In subsequent subcultures (II, III) some salt sensitive colonies were obtained either at 28° or 42°C for RCa-1 and some at 37° or 42°C for Ca-2. At neutral pH salt sensitive colonies were scored in subculture II and III especially at 28°C. The frequency of salt sensitive colonies was higher in subculture II for RCa-1 and in subculture III (42°C) for Ca-2. Some salt sensitive colonies were also scored at 37°C in subculture III with neutral pH. Hence in RCa-1 at neutral pH, high TMP concentration (175 $\mu\text{g ml}^{-1}$) in subculture II and alkaline pH at 150 $\mu\text{g ml}^{-1}$ TMP at 28°C or 125/175 $\mu\text{g ml}^{-1}$ at 42°C in subculture I, yielded highest proportion of salt sensitive colonies. In Ca-2 salt resistance was lost with high frequency at low TMP concentration in subculture II and with lesser frequencies at high TMP concentration which further declined in subculture III. Loss of salt tolerance was accomplished at non permissive temperature and low TMP concentration in neutral medium in subculture II which further raised to 100 percent in subculture III. Ca-2 rendered salt sensitive colonies both at low (I subculture), optimum (I, II) or high (III) temperature in alkaline medium (Table 1).

Randomly picked ten (or less in case where less number of salt sensitive colonies were obtained) salt sensitive colonies were checked for their plasmid loss by gel electrophoresis of total cell lysate. All showed deletions except 10 derivatives which exhibited complete plasmid loss (Table 2). Sizes of plasmids with deletions were smaller when compared with that of parental plasmid.

When the cured strains were examined for the effect of curing on Gram staining behaviour and their cell as well as colony morphology, striking differences were observed. Colony size of all derivatives of RCa-1 increased and their color changed from yellow to offwhite. Alignment of rods to filamentation was also recorded in derivatives of RCa-1. However Gram staining character was retained except that crystal violet was retained relatively more due to increased deposition of PG. In the derivatives of Ca-2 colony color changed to white only in case of Ca-2a2, while degeneration in rods of Ca-2b and Ca-2e was recorded.

None of cured derivatives could tolerate more than 0.5 M NaCl in liquid medium. For studying the impact of plasmid on Na⁺ uptake, parental strains and their cured derivatives were grown in 0.1 M NaCl L broth for 24 hours at 37°C and Na⁺ in the bacterial pellet was estimated. A significant increase in Na⁺ uptake by all cured

derivatives of RCa-1 (except RCa-1d) was displayed. However derivatives of Ca-2 behaved somewhat differently. Only Ca-2b showed exalted level of Na⁺ uptake, Ca-2a and Ca-2d exhibited lesser increase and slight decrease in rest of the derivatives was observed (Fig. 1).

To probe whether curing/treatment with TMP, pH, etc. have any effect on cell wall composition, teichoic acid, DAP and PG contents of the cell walls of parental strains and cured derivatives were determined. Increased incorporation of PG in walls of cured derivatives of both RCa-1 and Ca-2 (except Ca-2b) was observed. The increase was more pronounced in some of the derivatives when compared with respective parental strains (Fig. 2a). Generally increased DAP incorporation in cured derivatives of RCa-1, but decreased in those of Ca-2, were recorded. Nevertheless great variability was exhibited in DAP content of different cured derivatives (Fig. 2c). Amount of teichoic acid (TA) varied in different cured derivatives. Among plasmid free isolates of RCa-1 amount of TA decreased relative to parental strain. In RCa-1 decrease was not significant where as TA in other derivatives was almost negligible. In the cured derivatives of Ca-2, TA incorporation increased significantly, over parental strain, in Ca-2a1, Ca-2a2 and Ca-2b. In rest of the derivatives of Ca-2 the amount of TA was very low (Fig. 2b).

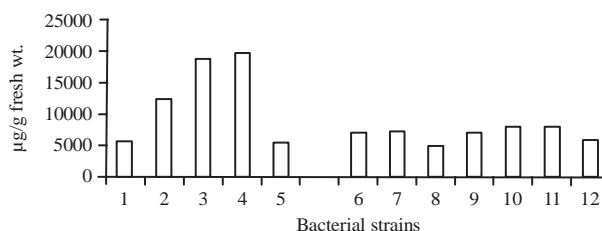


Fig. 1: Na uptake by salt tolerant parental strains and their plasmid free derivatives in 0.1 M NaCl supplemented L broth.

Discussion

Many workers have reported plasmid/s residing in salt tolerant /moderately halophilic bacteria (Hasnain and Taskeen, 1989; Sherwani and Hasnain, 1990, 1994; Fernandez-Castillo *et al.*, 1992; Vargas *et al.*, 1995; Mellado *et al.*, 1995, 1997; Hasnain and Thomas, 1996; Ventosa *et al.*, 1998; Mirza *et al.*, 1998). Plasmids are extrachromosomal autonomous DNA elements which are normally inherited stably in the next generation and can only be lost spontaneously from the host cells by replication error segregation (Old and Primrose, 1994). Plasmids bear certain genes for plasmid maintenance functions such as initiation and control of replication (Couturier *et al.*, 1988).

Bacteria utilize a number of mechanisms to maintain low copy number plasmids (Jensen and Gerdes, 1995; Holcik and Iyer, 1997). These can be divided into mechanisms that actively distribute the plasmid to daughter cells at cell division. Those which resolve oligomers (when they occur) into monomers and those which cause the death of plasmid free daughter cells by post segregational killing. In all cases of post segregational killing a plasmid encoded gene system produces a stable poison and an unstable antidote. In a plasmid deficient offspring a protein antidote is more readily digested by cellular proteases than the poison and the cell is killed by the toxic effects of the later (Falla and Chopra, 1999). One way of analysis of plasmid born characteristics is to cure plasmid from the bacterial strain and observe its impact. With the assumption

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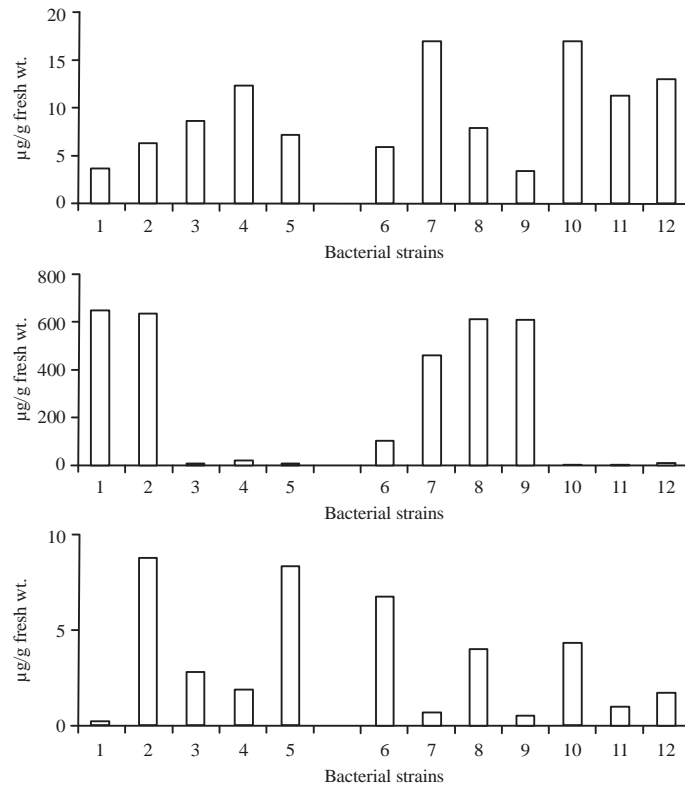


Fig. 2: Cell wall components of salt tolerant parental strains and their plasmid free derivatives a) peptidoglycan, b) teichoic acid, c) diamino pimelic acid. Bacterial strains were grown at 37°C and pH 7 for 24 hours on L agar and from the purified wall different components were determined.

Table 1: Percentage salt sensitive colonies screened by replica plating

Strains	pH	Temp	Subculture TMP ($\mu\text{g ml}^{-1}$)								
			I			II			III		
			125	150	175	125	150	175	125	150	175
RCa-1	6	28	-	-	-	-	-	-	-	-	-
		37	-	-	-	-	-	-	-	-	-
		42	-	-	-	-	-	-	-	-	-
	7	28	-	-	-	62.5	60 (33)	100	22	20	11
		37	-	-	-	-	-	-	6 (33)	15	7
		42	-	-	-	-	-	-	-	-	-
	9	28	90.9	100	84.6	-	-	-	10	-	-
		37	-	60	50	-	-	-	-	-	-
		42	100	80	100	15	12	-	4	-	3.3
	6	28	-	-	-	-	-	66	-	-	-
		37	-	-	-	-	-	-	-	-	-
		42	-	-	-	-	-	-	-	-	-
Ca-2	7	28	-	-	-	58	18	14	-	8	7
		37	-	-	-	-	-	-	14	15	7
		42	-	-	-	28	-	-	100	-	20
	9	28	19	-	7	-	-	-	-	-	-
		37	-	50	29	-	-	-	-	10	-
		42	-	-	-	-	-	-	3.6	-	3
	6	28	-	-	-	-	-	-	-	-	-
		37	-	-	-	-	-	-	-	-	-
		42	-	-	-	-	-	-	-	-	-

Note: Numbers in parenthesis indicate the total number of colonies obtained

Table 2: Cured derivatives of RCa-1 and Ca-2 along with the conditions where curing was accomplished

Cured derivatives	Complete Plasmid Loss
RCa-1a	175 µg ml ⁻¹ TMP, II, pH-7, 28°C
RCa-1b	125 µg ml ⁻¹ TMP, III, pH-9, 28°C
RCa-1c	150 µg ml ⁻¹ TMP, I, pH-9, 28°C
RCa-1d	125 µg ml ⁻¹ TMP, II, pH-9, 42°C
Ca-2a1	175 µg ml ⁻¹ TMP, II, pH-6, 28°C
Ca-2a2	175 µg ml ⁻¹ TMP, II, pH-6, 28°C
Ca-2b	125 µg ml ⁻¹ TMP, III, pH-7, 42°C
Ca-2c	150 µg ml ⁻¹ TMP, III, pH-9, 37°C
Ca-2d	175 µg ml ⁻¹ TMP, I, pH-9, 37°C
Ca-2e	175 µg ml ⁻¹ TMP, I, pH-9, 37°C

that salt tolerant property may be associated with plasmid/s residing in RCa-1 and Ca-2, curing experiments were performed.

Curing agents can interfere plasmid replication mechanisms. In the absence of a universally effective curing agent, combinations of different physical and chemical curing agents may be examined for their curing efficiency. Treatment of bacterial strains with various curing agents could promote plasmid loss (Stanisich, 1988; Lakshmi *et al.*, 1988; Saint and Venables, 1990; Debbia, 1992; Crosa *et al.*, 1994). Yasmin and Hasnain (1996) have reported TMP as a successful curing agent, hence curing was accomplished with it. For determination of effective concentration of TMP at which plasmid loss can be promoted, minimum inhibitory concentration (MIC) of TMP was worked out which was 80 µg ml⁻¹. Keeping in view the MIC of TMP, three different concentrations of TMP were selected and applied in combination with different temperatures and pH conditions. Plasmid loss/deletion rates have also been reported to be governed by the type of substrate and pH of growth medium (Lakshmi *et al.*, 1988, 1989; Saint and Venables, 1990). Different combinations of TMP conc. with temperature and pH conditions yielded different results. At pH 7 salt sensitive colonies were attained in subculture II but at alkaline pH (9) strains rendered plasmid free derivatives in the subculture I with high frequency. Curing efficiency (complete loss/deletions) varied from 3 to 100 per cent in these strains. This could possibly be attributed to the plasmid and particularly to the bacterial host carrying plasmid (Stanisich, 1988). Present results demonstrated that curing efficiency is also influenced by environmental conditions. Plasmid loss did not have much impact on the colony morphology except that colony colours of all derivatives of RCa-1 and Ca-2a1 of Ca-2 were changed. Cell morphology of plasmid free strains (RCa-1b, RCa-1c, FiCa-1d, Ca-2a1, Ca-2a2) showed difference from the parental strains. All plasmid free derivatives of RCa-1 exhibited slight elongation in the sizes of the rods which displayed tendency towards chain formation. Morphological changes in cured strains have also been reported by other workers (Yasmin and Hasnain, 1996; Muzzamal, 1998). Cell elongation followed by asymmetrical cell division plays an important role in determining plasmid curing. Curing process is a result of plasmid segregation into minicell like elements (Debbia, 1992).

High temperature has been found to be an effective curing agent. However results of our study revealed that low temperature (28°C) proved effective as curing agents at some TMP concentrations and pHs (RCa-1a-175 µg ml⁻¹ TMP, II, pH 7, 28°C; RCa-1b- 125 µg ml⁻¹ TMP, III, pH 9, 28°C; RCa-1c - 150 µg ml⁻¹ TMP, I, pH 9, 28°C; Ca-2a1 - 175 µg ml⁻¹ TMP, II, pH 6, 28°C; Ca-2a2 - 175 µg ml⁻¹ TMP, II, pH 6, 28°C). Nevertheless higher temperature (42°C) also proved successful in curing these plasmids (RCa-1d - 125 µg ml⁻¹ TMP, II, pH 9, 42°C; Ca-2b - 125 µg ml⁻¹ TMP, III, pH 7, 42°C). Generally increased Na uptake by plasmid free strains of RCa-1 may be ascribed to the loss of some efficient check/control on Na

uptake, with the loss of plasmid. Na uptake ability in three cured derivatives of Ca-2 (Ca-2a1, Ca-2b, Ca-2d) was also increased with plasmid removal. Hence plasmid might have stringent control on the uptake of Na, maintaining the low osmolality in the cell.

Some constituents of cell wall were also analysed to determine if curing had any impact on them. Diaminopimelic acid, peptidoglycan and teichoic acid were analysed henceforth. Parental and plasmid free strains were compared for their cell wall characteristics. Plasmid curing depends on size of plasmid, cell shape and cell growth which in turn are maintained by PG synthesis, glycan chain geometry and orientation (Gally and Cooper, 1993; Nanninga, 1998). Variability in size and shape of cell, exhibited with extreme of environmental changes, alters PG content (Sabri and Hasnain, 1996). Importance of mechanical interactions among PG in determining normal cell shape has also been reported (Levin *et al.*, 1998; Nanninga, 1998). PG content in cured derivatives of Ca-2 and RCa-1 showed marked increase (except Ca-2b) whilst for other wall constituents, variation was manifested in different plasmid free derivatives. Increased incorporation of DAP directly reflects extension of stress bearing cell surface (Woldringh *et al.*, 1987; Nanninga, 1998). It seems as if temperature, pH and curing agent alter arrangement of teichoic acid. Arrangement of teichoic acid and other component polymers within wall and at its surface is affected by its ionic environment (Archibald, 1988; Hancock, 1994). Sabri and Hasnain (1996) have also reported decreased teichoic acid at alkaline pH and high temperature in cell division mutants. At alkaline pH (9) marked decrease in teichoic acid contents in RCa-1b, RCa-1c, RCa-1d, Ca-2c, Ca-2d & Ca-2e is recorded. All of these were derived at alkaline pH and high temperature. Mauel *et al.* (1995) have reported that extremes of pH also affect the transcription of genes responsible for teichoic acid synthesis. Hence stress environment evidently affect plasmid loss and efficiency of plasmid loss is better at sub optimal temperature and/or alkaline pH.

References

- Archibald, A.R., 1988. Bacterial Cell Wall Structure and the Ion Environment. In: Homeostatic Mechanism and the Ion Environment, Whittenburg, R., G.W. Gould, J.G. Baker and R. Board (Eds.). Bath University Press, UK., pp: 37-51.
- Caro, L., G. Churchward and M. Chandler, 1984. Study of plasmid replication *in vivo*. *Methods Microbiol.*, 17: 97-122.
- Couturier, M., F. Bex, P.L. Bergquist and W.K. Maas, 1988. Identification and classification of bacterial plasmids. *Microbiol. Rev.*, 52: 375-395.
- Crosa, J.H., M.E. Tolmasky, L. Actis and S. Falkow, 1994. Plasmids. In: *Methods for General and Molecular Bacteriology*, Gerhardt, P., R.G.S. Murray, W.A. Woad and N. Krieg (Eds.). American Society for Microbiology, Washington, DC., pp: 365-386.
- Debbia, E.A., 1992. Filamentation promotes F'lac loss in *Escherichia coli* K12. *Microbiology*, 138: 2083-2091.
- Diver, J.M. and R. Wise, 1986. Morphological and biochemical changes in *Escherichia coli* after exposure to ciprofloxacin. *J. Antimicrob. Chemother.*, 18: 31-41.
- Falla, T.J. and I. Chopra, 1999. Stabilization of *Rhizobium symbiosis* plasmids. *Microbiology*, 145: 515-516.
- Fernandez-Castillo, R., C. Vargas, J.J. Nieto, A. Ventosa and F. Ruiz-Berraquero, 1992. Characterization of a plasmid from moderately halophilic eubacteria. *Microbiology*, 138: 1133-1137.
- Furman, N.H., 1975. *Standard Methods of Chemical Analysis*. 6th Edn., Krieger Publishing Company, New York.
- Gally, D. and S. Cooper, 1993. Peptidoglycan synthesis in *Salmonella typhimurium* 2616. *Microbiology*, 139: 1469-1476.
- Gerhardt, P., R.G.E. Murray, W.A. Wood and N.R. Krieg, 1994. *Methods for General and Molecular Bacteriology*. 2nd Edn., American Society for Microbiology, Washington, DC., ISBN: 9781555810481, pp: 343.

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- Hancock, I.C., 1994. Analysis of Cell Wall Constituents of Gram Positive Bacteria. In: Chemical Methods in Prokaryotic Systematics, Goodfellow, M. and A.G. O'Donnell (Eds.). John Wiley and Sons Ltd., New York, ISBN: 9780471941910, pp: 63-84.
- Hasnain, S. and C.M. Thomas, 1996. Two related rolling circle replication plasmids from salt-tolerant bacteria. *Plasmid*, 36: 191-199.
- Hasnain, S. and N. Taskeen, 1989. Characterization of salt tolerant bacteria isolated from the rhizosphere of *Leptochloa fusca* and *Atriplex rhocodoides*. *Pak. J. Pharm. Sci.*, 2: 47-53.
- Holcik, M. and V.M. Iyer, 1997. Conditionally lethal genes associated with bacterial plasmids. *Microbiology*, 143: 3403-3416.
- Hooper, D.C., J.S. Wolfson, G.L. McHugh, M.D. Swartz, C. Tung and M.N. Swartz, 1984. Elimination of plasmid pMG110 from *Escherichia coli* by novobiocin and other inhibitors of DNA gyrase. *Antimicrob. Agents Chemother.*, 25: 586-590.
- Jensen, R.B. and K. Gerdes, 1995. Programmed cell death in bacteria: Proteic plasmid stabilization systems. *Mol. Microbiol.*, 17: 205-210.
- Lakshmi, V.V. and C.M. Thomas, 1996. Curing of F-like plasmid TP181 by plumbagin is due to interference with both replication and maintenance functions. *Microbiology*, 142: 2399-2406.
- Lakshmi, V.V., P. Sridhar and H. Polasa, 1989. Loss of plasmid linked antibiotic resistance in *Escherichia coli* on treatment with some phenolic compounds. *FEMS Microbiol. Lett.*, 57: 275-278.
- Lakshmi, V.V., P. Sridhar, B.T. Khan and H. Polasa, 1988. Mixed-ligand complexes of platinum (II) as curing agents for pBR322 and pBR329 (ColE1) plasmids in *Escherichia coli*. *Microbiology*, 134: 1977-1981.
- Lebrun, M., J. Loulergue, E. Chaslus-Dancla and A. Audurier, 1992. Plasmids in *Listeria monocytogenes* in relation to cadmium resistance. *Applied Environ. Microbiol.*, 58: 3183-3186.
- Leduc, M., C. Crehel, E. Siegel and J. van Heijenoort, 1989. Multilayered distribution of peptidoglycan in the periplasmic space of *Escherichia coli*. *Microbiology*, 135: 1243-1254.
- Levin, P.A., J.J. Shim and A.D. Grossman, 1998. Effect of *minCD* on FtsZ ring position and polar septation in *Bacillus subtilis*. *J. Bacteriol.*, 180: 6048-6051.
- Mauel, C., A. Bauduret, C. Chervet, S. Beggah and D. Karamata, 1995. In *Bacillus subtilis* 168, teichoic acid of the cross-wall may be different from that of the cylinder: A hypothesis based on transcription analysis of tag genes. *Microbiology*, 141: 2379-2389.
- Mellado, E., J.A. Asturias, J.J. Nieto, K.N. Timmis and A. Ventosa, 1995. Characterization of the basic replicon of pCM1, a narrow-host-range plasmid from the moderate halophile *Chromohalobacter marismortui*. *J. Bacteriol.*, 177: 3443-3450.
- Mellado, E., M.T. Garci, J.J. Nieto, S. Kaplan and A. Ventosa, 1997. Analysis of the genome of *Vibrio costicola*: Pulsed-field gel electrophoretic analysis of genome size and plasmid content. *Syst. Applied Microbiol.*, 20: 20-26.
- Mirza, T.Z., A.N. Sabri and S. Hasnain, 1998. Salt tolerant bacteria from rhizosphere, rhizoplane, histoplane and phylloplane of Mazus plant inhabitant of salt range. *Sci. Int.*, 10: 151-156.
- Muzzamal, S., 1998. Efficiency of curing agents for plasmid removal from salt tolerant bacteria isolated from *Desmostachya bipinnata*. M.Sc. Thesis, Punjab University, Lahore, Pakistan.
- Nanninga, N., 1998. Morphogenesis of *Escherichia coli*. *Microbiol. Mol. Biol. Rev.*, 62: 110-129.
- Old, R.W. and S.B. Primrose, 1994. Principles of Gene Manipulation: An Introduction to Genetic Engineering. Blackwell Scientific Publications, USA., pp: 42.
- Qazi, S.H.A. and S. Hasnain, 1997. Plasmid associated salt tolerance property of *Agrobacterium tumefaciens* strains from plants of Punjab, Pakistan. *Pak. J. Zool.*, 29: 7-13.
- Sabri, A.N. and S. Hasnain, 1996. Polypeptide products in diviva+ and diviva-strains of *Bacillus subtilis* under varying temperature, pH and stavation conditions. *Pak. J. Zool.*, 28: 303-309.
- Saint, C.P. and W.A. Venables, 1990. Loss of Tdn catabolic genes by deletion from and curing of plasmid pTDN1 in *Pseudomonas putida*: Rate and mode of loss are substrate and pH dependent. *Microbiology*, 136: 627-636.
- Sherwani, S.K. and S. Hasnain, 1990. Alkaliphilic moderately halophilic plasmid harbouring gram-negative bacteria from saline sodic soils. *Sci. Int.*, 2: 147-150.
- Sherwani, S.K. and S. Hasnain, 1994. Molecular Genetic Studies on Osmoregulation of Halotolerant Bacteria Isolated from the Rhizosphere of *Sueda fruticosa* and *Sporobolus pelidis*. In: Current Development in Salinity and Drought Tolerance of Plants, Naqvi, S.S., R. Ansari, T.J. Flower and A.R. Azmi (Eds.). AEARC., Tandojam, pp: 198-206.
- Stanisich, V.A., 1988. Identification and Analysis of Plasmids at the Genetic Level. In: Methods in Microbiology, Grinsted, J. and P.M. Bennett (Eds.). 2nd Edn., Academic Press, New York, pp: 11-47.
- Thomas, C.M., 1984. Analysis of Clones. In: Methods in Microbiology, Bennett, P.M. and J. Grinsted (Eds.). Academic Press, London, pp: 163.
- Vargas, C., R. Fernandez-Castillo, D. Canovas, A. Ventosa and J.J. Nieto, 1995. Isolation of cryptic plasmids from moderately halophilic eubacteria of the genus *Halomonas*. Characterization of a small plasmid from *H. elongata* and its use for shuttle vector construction. *Mol. Gen. Genet.*, 246: 411-418.
- Ventosa, A., J.J. Nieto and A. Oren, 1998. Biology of moderately halophilic aerobic bacteria. *Microbiol. Mol. Biol. Rev.*, 62: 504-544.
- Woldringh, C.L., P. Huls, E. Pas, G.J. Brakenhoff and N. Nanninga, 1987. Topography of peptidoglycan synthesis during elongation and polar cap formation in a cell division mutant of *Escherichia coli* MC4100. *Microbiology*, 133: 575-586.
- Work, E., 1957. Reaction of ninhydrin in acid solution with straight-chain amino acids containing two amino groups and its application to the estimation of $\alpha\epsilon$ -diaminopimelic acid. *Biochem. J.*, 67: 416-423.
- Yasmin, A. and S. Hasnain, 1996. Plasmid curing with TMP from salt tolerant *Pseudomonads*. *Proc. Pak. Congr. Zool.*, 16: 221-229.
- Yasmin, A. and S. Hasnain, 1998. Altered Gram-staining property and some cell wall constituents of bacteria under NaCl stress. *Biologia*, 44: 291-297.