Rpb4p Is Necessary for RNA Polymerase II Activity at High Temperature*

(Received for publication, April 1, 1999)

Isabelle Maillet, Jean Marie Buhler, André Sentenac, and Jean Labarre‡

From the Service de Biochimie et Génétique Moléculaire, CEA-Saclay, F-91191 Gif-sur-Yvette Cedex, France

Rpb4p and Rpb7p are two subunits of the yeast RNA polymerase II, which form a subcomplex that can dissociate from the enzyme in vitro. Whereas RPB7 is essential, RPB4 is dispensable for cellular viability. However, the rpb4 null mutant is heat-sensitive, and it has been suggested that Rpb4p is an essential component for cellular stress response. To examine this hypothesis, we used two-dimensional gel electrophoresis to analyze the protein expression pattern of the rpb4 null mutant in response to heat shock, oxidative stress, osmotic stress, and in the post-diauxic phase. We show that this mutant is not impaired in stress induced transcriptional activation: the absence of heat shock response of the mutant is due to a general defect in RNA polymerase II activity at high temperature. Under this condition, Rpb4p is necessary to maintain the polymerase activity in vitro. The heat growth defect of the rpb4 null mutant can be partially suppressed by overexpression of RPB7, suggesting that Rpb4p maintains or stabilizes Rpb7p in the RNA polymerase. We also demonstrate that rpb4 null mutant is an appropriate tool to analyze the involvement of transcriptional events in the survival and adaptation to heat shock or other stresses.

The yeast RNA polymerase II (Pol II),1 which catalyzes the transcription of all protein-encoding genes, is composed of 12 subunits. Two of them, Rpb4p and Rpb7p, are closely associated in a subcomplex denoted Rpb4/7 that can be dissociated from the polymerase under nondenaturing conditions or by mild urea treatment (1). Rpb4/7 is also lacking in Pol II enzymes purified from the rpo B1 mutant (1) and from the rpb4 null mutant (2). The physical interaction between the two subunits was confirmed by two-hybrid experiments (3). Structural data obtained by electron microscopy of the complete RNA polymerase and the enzyme devoid of the Rpb4/7 subcomplex have located these two subunits in the floor of the DNA-binding cleft (4). The function of this subcomplex could be to induce a tight closure of the arm domain surrounding the cleft and to favor the transition from an “open” conformation of the polymerase during the initiation step of transcription to a “closed” conformation during the elongation step (5).

Whereas RPB7 is essential, RPB4 is not during normal growth conditions. Strains carrying a deletion of RPB4 are cold- and heat-sensitive and auxotrophic for inositol (6). Rpb4

null mutant cells are unable to transcribe HSP26 and SSA3 mRNA under heat shock condition and when cells enter stationary phase (7). It has been argued that this reflects specialized regulatory role of Rpb4p, which would endow Pol II with the ability to respond to stress stimuli in vitro (7). Alternatively, the role of Rpb4p may be to prevent the inactivation of the polymerase under stress conditions. This second hypothesis is supported by in vitro experiments showing that Rpb4p is required for Pol II activity at temperature extremes (10 and 35 °C) (8). This would imply that the growth pattern of rpb4 null mutants is due to a general Pol II defect but not to a modification of its regulatory properties.

To settle the issue, we used the two-dimensional gel electrophoresis approach and examined the protein expression profile of a rpb4 null mutant in response to various stress conditions. We show that this mutant is not impaired in stress induced transcriptional activation but is globally defective in Pol II activity at high temperatures. Our data suggest that a role of Rpb4p would be to maintain or stabilize Rpb7p in the enzyme.

MATERIALS AND METHODS

Bacteria and Yeast Strains—The Escherichia coli strain XLIblue (upE44, lacIq, recA1, endA1, gyrA46, thiI, relA1, lacI[F(proAB’), lacP’], lacZA15, Tn10(tet')] was used as a host for the cloning construction. The Saccharomyces cerevisiae strains RY260 (MATa ura3–52 rpb1–1), SUB62 (MATa lys2–801 leu2–3,12–112 ura3–52 his3–Δ200 trp1–1am) and its isogenic derivative MC11 (MATa lys2–801 leu2–3,12–112 ura3–52 his3–Δ200 trp1–1am rpb4–HIS3) were described previously (7, 9).

Culture Conditions—Cells were grown at 24 °C in 2.5 ml of minimal medium containing 0.67% yeast nitrogen base minus amino acids (Difco), 2% glucose, and buffered at pH 5.8 with 1% succinate and 0.6% NaOH. When needed, amino acids (30 mg/liter) were added to the culture medium. To study the post-diauxic phase, cells were grown at 24 °C in a supplemented minimal medium (YNBS) as described by Boy-Marcotte et al. (10).

Cell Labeling and Two-dimensional Gel Electrophoresis—2 ml of mid-log cells (A600, 0.3) were exposed to 0.4 mM H2O2 (oxidative stress) or 0.7 M NaCl (osmotic stress) or 38 °C (heat shock) for 30 min, collected at t = 0. Cells were then labeled with 350 μCi of [35S]methionine at t = 15 min, collected at t = 30 min, and processed for two-dimensional gel electrophoresis. Post-diauxic phase study, log phase cultures were monitored for growth and for glucose concentration in the medium, until they entered the post-diauxic shift phase. Then, 2 ml of the culture (A600, 6–7) were labeled for 45 min with 300 μCi of [35S]methionine and processed for two-dimensional gel electrophoresis. Preparation of cell extracts and two-dimensional gel electrophoresis were performed as described previously (11).

Sensitivity to Heat Shock and H2O2—Mid-log phase cultures grown in minimal medium at 24 °C were subjected to: 1) a shift to 38 °C for 60 min (mild heat shock) or 2) a shift to 50 °C for 15 min (severe heat shock) or 3) a shift to 38 °C and after 5 min were treated with 1 mM or 3 mM H2O2 for 60 min (oxidative stress) or 4) no shift and no treatment (control). Aliquots were then removed and shifted at 24 °C and serially diluted (10-fold at each step). Five μl were spotted onto rich broth (YPD) plates and incubated at 25 °C to measure colony-forming units.

For adaptation studies, cells were subjected to a shift to 38 °C for 60 min and then to a shift to 50 °C for 15 min (thermotolerance assay) or to a treatment at 38 °C with 3 mM H2O2 or 60 min (adaptation to H2O2).
Rpb4p Is Not Involved in General Stress Response

Cells were then shifted to 24 °C and processed as described above to determine colony-forming units.

Plasmid Construction—pYX212 is a 2-μm URA3 vector with TPI promoter, one of the strongest constitutive promoters in yeast (Ingenius). To construct pYX212-Rpb7, oligonucleotides (Rpb7-L: 5′ TTA CGG ATC CAT GTT TTT TAT TAA AGA CCT TTC GCT TA 3′ and Rpb7-L: 5′ GAC TAT GCT CGA GTT AAA TAG CAC CCA AAT ATT CTT T T 3′) were used to add a 5′ BamHI site and a 3′ XhoI site (underlined) to the Rpb7 coding sequence amplified from the genomic DNA of strain X2180. This fragment was directionally cloned between the BamHI and XhoI sites of pYX212. The initiator (ATG) and stop (TAA) codons of the cloned RPB7 sequence are italic. For suppression experiments, strains MC11 and SUB62 were transformed by standard methods (12) either with the vector pYX212 or with the plasmid pYX212-Rpb7, streaked on rich broth (YPD) plates, and incubated at 24, 34, or 37 °C for 2–4 days.

Northern Blot Analysis—Mid-log cells (A∞500 0.3) grown at 24 °C were shifted to 38 °C at t = 0 and collected for mRNA preparation at t = 0, 15, and 60 min. For each time point, 6 × 107 cells were collected by centrifugation, and total mRNA was prepared by hot phenol extraction method of Schmitt (13). RNA concentration was determined by spectrophotometry at A260 and A230. 20 μg RNA was mixed with 6 μl glyoxal, 0.1 M NaPO4, pH 7.0, and Me2SO, denatured and subjected to electrophoresis in a 1.2% agarose gel containing 10 mM NaPO4. RNA was blotted onto positively charged nylon membrane (Roche Molecular Biochemicals) in 10 × SSC (1 × SSC is 0.15 M NaCl and 0.015 M sodium citrate) by using a vacuum blotter (Appligene-Oncor). The membrane was dried at 65 °C, and RNA was UV-cross-linked to the membrane (Stralinker 1800; Stratagene). The blot was hybridized first with a mixture of ACT1 and DED1 probes, second with STE2 probe and third with the 25 S probe.

Probes were synthesized by polymerase chain reaction of genomic DNA using oligonucleotide primers: for ACT1 probe, 5′ TTT TGG GGT GCC CCA GAA GAA CAC C 3′; for DED1 probe, 5′ GCC GAA AAA TTT AAG CAT CAA CGA CCA C 3′; for STE2 probe, 5′ GGC ACC GAT TCC TTT CTA GGA GTA AAA G 3′; and for TATA box, 5′ CTC ACC GCA ATT TCC TTC GCT CA 3′. The probes were labeled with [α-32P]dNTP by random priming using the Megaprime Labeling System RPN1606 (Amersham Pharmacia Biotech).

The synthetic oligonucleotide GCCATTCTACAAACGAAGCCGACTC, used as a probe for 25 S rRNA expression, was radioactively labeled using T4 polynucleotide kinase and [γ-32P]ATP.

RESULTS AND DISCUSSION

Previous works have shown that the rpb4 null mutant strain (devoid of the fourth subunit of Pol II) is temperature-sensitive (14) and does not express HSP26 and SSA3 mRNA under heat shock condition and when cells enter stationary phase (7) suggesting that the rpb4 null strain could be defective in general stress response. We used the two-dimensional gel electrophoresis approach to analyze the expression of heat shock proteins in the wild type (SUB62) and rpb4 null (MC11) strains. Cells were pulse-labeled with [35S]methionine for 15 min at 24 °C or after a temperature shift from 24 to 38 °C, and protein extracts were subjected to comparative two-dimensional gel electrophoresis. The protein expression pattern of the two strains grown at 24 °C was identical (Fig. 1A, lanes 2 and 4, not shown), indicating that there is no expression defect of rpb4 null strain at 24 °C. In contrast, the two-dimensional maps from the two heat shocked strains were very different. More than 50 heat shock proteins (for example, the aldehyde dehydrogenase Ald5p and the heat shock proteins Hsp82p and Hsp104p) were strongly induced in the wild type strain, and no significant induction was observed in the mutant (compare Fig. 1, B and C). This observation includes proteins whose expression is dependent on Hsf1p and Msn2/4p transcription factors known to be involved in heat shock response (31). Moreover, the proteins usually repressed under heat shock conditions (for example the ribosomal proteins and translation factors like Yef3p) were no longer repressed in the mutant strain. These results extended previous results (7) showing that the rpb4 null mutant strain is impaired in the global heat shock response.

We then analyzed the response of rpb4 null mutant to oxidative stress, osmotic stress, and diauxic phase transition. When wild type cells were treated with 0.4 mM H2O2, more than 100 oxidative stress-responsive proteins were observed on two-dimensional gels as described previously (15). No defect in H2O2 response was evidenced in rpb4 null strain (Fig. 2A). For example, the TRR1-encoded thioredoxin reductase, the CCP1-encoded cytochrome c peroxidase, or YOL151W were similarly induced in both strains. Similarly, the osmotic stress response was identical in both the wild type and mutant strains (Fig. 2B). For example, enzymes of the glycerol biosynthesis pathway (glycerol phosphate dehydrogenase Gpd1p and glycerol phosphate phosphatases Gpp1p and Gpp2p), which are essential in the osmotic stress response (16), were induced to the same level in both strains. Finally, there was no significant difference in protein expression between the two strains in post-diauxic phase condition (Fig. 2C); in both strains, the expression of the glycolytic enzymes such as Eno2p and Fba1p were shut down and enzymes involved in respiratory metabolism such as Aca1p and Mdh1p were normally induced. Moreover, stress proteins such as the glutathione reductase Ggr1p and the catalase Ctt1p were similarly induced.

![FIG. 1. Comparative two-dimensional gel electrophoresis of proteins extracted from the wild type, rpb4, and rpb1-1 mutant strains in heat shock condition.](image-url)
These results indicate that the rpb4 null strain, which is defective in heat shock response, is not impaired in other stress responses at 24 °C. To confirm this point, we tested whether the mutant showed a normal oxidative stress response at 38 °C. The cells were shifted from 24 to 38 °C and after 5 min were treated with 0.4 mM H2O2. When this treatment was applied to the wild type strain, a strong induction of both heat shock and oxidative stress proteins was observed. In sharp contrast, no induction of either the heat shock proteins or the oxidative stress proteins was evidenced in the rpb4 null mutant (data not shown). The fact that the oxidative stress response which was unaffected at 24 °C became defective at 38 °C suggested that the ability of the mutant to respond to stress was suppressed at high temperatures.

We reasoned that a thermosensitive Pol II mutant would probably have a deficiency in heat shock response similar to the defect observed in rpb4 null strain. To test this hypothesis, we analyzed the heat shock response of a well characterized Pol II mutant, rpb1-1 (strain RY 260), which affects the largest Pol II subunit and stops rapidly mRNA synthesis when shifted to 37 °C (9). The relevant two-dimensional gel electrophoresis is shown in Fig. 1C. No heat shock protein was induced after the temperature shift, indicating that the defect in mRNA synthesis appears quickly after the shift to the restrictive temperature. Moreover, proteins like Yef3p, which are repressed in the wild type strain, were not repressed in rpb1–1 strain as in the case of the rpb4 null strain (Fig. 1C). Thus, the protein expression pattern of the two pol II mutants rpb4 null and rpb1–1 under heat shock condition are strikingly similar, suggesting that rpb4 null mutant, like rpb1–1 has a general transcriptional defect at the restrictive temperature.

A more direct method to test the functional defect of Pol II consists to analyze the in vivo Pol II activity after transfer to restrictive temperature. A mutant having a defective Pol II subunit and stops rapidly mRNA synthesis when shifted to 37 °C (9). The relevant two-dimensional gel electrophoresis is shown in Fig. 1D. No heat shock protein was induced after the temperature shift, indicating that the defect in mRNA synthesis appears quickly after the shift to the restrictive temperature. Moreover, proteins like Yef3p, which are repressed in the wild type strain, were not repressed in rpb1–1 strain as in the case of the rpb4 null strain (Fig. 1C). Thus, the protein expression pattern of the two pol II mutants rpb4 null and rpb1–1 under heat shock condition are strikingly similar, suggesting that rpb4 null mutant, like rpb1–1 has a general transcriptional defect at the restrictive temperature.
rRNA, which is a Pol I transcript not immediately affected by a Pol II defect (9). The results showed that *DED1* and *STE2* mRNAs decreased rapidly after the shift, consistent with their short half-lives (9, 17); *ACT1* mRNA levels decreased more slowly with a deduced half-life of about 30 min. The *rpb1-1* strain behaved similarly. In contrast, in the wild type control experiment, mRNA level decreased slightly 15 min after the temperature shift and resumed to the basal level after 1 h. Thus, the rapid decrease of these three individual mRNAs in the mutant strains demonstrates that these mRNAs are no longer synthesized after the temperature shift. This result can be extended to all mRNAs, since Choder and Young (7) analyzed total poly(A)^+ RNA and showed that the *rpb4 null* mutant rapidly ceases global mRNA synthesis after a shift to 37 °C. Altogether, these data showing arrest of individual and global mRNA synthesis demonstrate that it is the activity of Pol II that is temperature-sensitive in the *rpb4 null* mutant. This result is also consistent with experiments showing that Pol II activity of this mutant is temperature-sensitive *in vitro* (8).

The *rpb4 null* mutant is not impaired in stress response, but has a Pol II defective at restrictive temperature. Usually, a mutation in an essential gene can generate a conditional defect in the function of the encoded protein. This general case is exemplified by the *rpb1-1* mutant. Paradoxically, in this work, the conditional defect of Pol II is due to the absence of a polymerase subunit. This defect can be due either to the heat inactivation of the polymerase or to the degradation of the polymerase devoid of Rpb4p when subjected to high temperature. This last possibility can be excluded, since after heat inactivation for 15 min, Pol II can regain full transcriptional activity in less than 10 min after the temperature is shifted back to 24 °C as demonstrated by the rapid recovery of *DED1* mRNA synthesis (data not shown). Rpb4p has then no role in preventing Pol II degradation but is probably required to prevent the inactivation of the enzyme or of one of its essential subunits. Rpb4p has been shown to interact with Rpb7p by biochemical and two-hybrid experiments (1, 3). Moreover, Edwards et al. (2) showed that Rpb7p subunit dissociated from Pol II when the polymerase was purified from *rpb4 null* extracts, suggesting that a role of Rpb4p is to stabilize Rpb7p in the Pol II. According to this hypothesis, at permissive temperature, Rpb7p would continue to interact weakly with Pol II in the absence of Rpb4p; at restrictive temperatures, the interaction between Rpb7p and Pol II would become too weak, leading to the dissociation of Rpb7p and to the inactivation of the enzyme. In that case, the overexpression of Rpb7p would compensate its weak interaction with the defective Pol II and rescue its activity. To examine this possibility, we overexpressed Rpb7p on a 2-μm plasmid in the *rpb4 null* strain and tested the temperature sensitivity of this strain (Fig. 4); whereas the *rpb4 null* strain was unable to grow at 33 and 34 °C, its growth resumed at these temperatures when Rpb7p is overexpressed. The suppression was partial, since Rpb7p overexpression did not allow growth of *rpb4 null* strain at higher temperatures (data not shown). These data are consistent with a role for Rpb4p in the stabilization of Rpb7p-Pol II interactions. Thus, Rpb4p would play a structural role in maintaining Rpb7p in the complex. Functionally, Rpb4p may act as a specific chaperone for Pol II. However, whereas chaperones are generally induced by heat shock condition, *RPB4* is neither induced in heat shock condition nor in any other stress conditions tested (18, 19).

Due to its conditional defect in mRNA transcription, the *rpb4 null* mutant can be used, as *rpb1-1*, to measure mRNA half-lives (20–22). Interestingly, deleting *RPB4* gene in a specific genetic background is much easier and less time consuming than the construction of the *rpb1-1* mutation. Moreover, since the heat inactivation of Pol II from *rpb4 null* mutant is reversible *in vivo*, the mutant could be an interesting tool to analyze the cellular response to environmental modifications in absence of Pol II transcription and then in absence of synthesis of responsive proteins. This type of analysis usually requires the use of transcriptional or translational inhibitors that may interfere with the cellular response investigated (20, 23). For example, we analyzed the sensitivity of *rpb4 null* strain to a severe heat shock (shift from 24 to 50 °C for 15 min) and found no significant difference with the wild type strain (Table I). In the same way, the mutant transferred to 38 °C is as resistant to oxidative stress (1 or 3 mM H₂O₂ for 1 h) as the wild type strain. These data indicate that the cell survival to these stresses is not dependent upon transcription and induction of stress proteins. Concerning heat shock, this conclusion is in agreement with previous results obtained with cycloheximide, a translational inhibitor (24). However, in the case of oxidative stress, this finding seems inconsistent with the property of the strain deprived of the transcriptional factor Yap1p: this mutant is unable to induce a part of the H₂O₂ stimulon (25) and is sensitive to H₂O₂ (26), whereas the *rpb4 null* strain, which is impaired in the induction of the entire stimulon, is not sensitive. This paradox could be explained knowing that the basal amount of proteins with antioxidant and protective functions (Ahp1p, Sod1p, Ssa1p, Trt1p, Tsa1p, Zw1p) is lower in the *yap1 null* mutant than in the wild type (27) and the *rpb4 null* strains. We also examined if the *rpb4 null* strain is able to acquire resistance to high temperature by pretreatment with a mild heat shock (thermotolerance). As shown in Table I, this property of the wild type strain is partly impaired in the *rpb4 null* strain, indicating that full thermotolerance requires the synthesis of a new set of proteins. Accordingly, previous reports have demonstrated that the expression of one protein, Hsp104p, is essential in this process (28–30). We also showed that a mild heat shock allowed wild type cells to survive to 3 mM H₂O₂ (23) but this adaptation property was lost in the mutant. Then, whereas resistance to H₂O₂ does not necessitate any transcriptional event, the process allowing cells to adapt to more severe oxidative stresses does require an adequate transcriptional response. This strongly suggests that the capacity of cells to survive stresses is mainly dependent upon the amount of protective proteins present in the cells before the stress and that the gene expression induced by the stress aims essentially at protecting the cells toward foreseeable more adverse conditions.
Acknowledgments—We thank M. Choder for strains; E. Boy-Marcotte, P. Thuriaux, and M. Toledano for support and critical reading of the manuscript; M. Siaut, M. Goussot, M. Saraiva, A. M. Deschampsme, S. Labarre, and G. Lagniel for support and encouragements.

REFERENCES

1. Ruet, A., Sentenac, A., Fromageot, P., Winsor, B., and Lacroute, F. (1980) J. Biol. Chem. 255, 6450–6455
2. Edwards, A. M., Kane, C. M., Young, R. A., and Kornberg, R. D. (1991) J. Biol. Chem. 266, 71–75
3. Khazak, V., Sadhale, P. P., Woychik, N. A., Brent, R., and Golemis, E. A. (1995) Mol. Biol. Cell 6, 759–775
4. Jensen, G. J., Meredith, G., Bushnell, D. A., and Kornberg, R. D. (1998) EMBO J. 17, 2353–2358
5. Asturias, F. J., Meredith, G. D., Poglitsch, C. L., and Kornberg, R. D. (1997) J. Mol. Biol. 272, 536–549
6. Rpb4p Is Not Involved in General Stress Response
7. Woychik, N. A., and Young, R. A. (1990) Trends Biochem. Sci. 15, 347–351
8. Choder, M., and Young, R. A. (1993) Mol. Cell. Biol. 13, 6984–6991
9. Rosenheck, S., and Choder, M. (1998) J. Bacteriol. 180, 6187–6192
10. Boy-Marcotte, E., Tadi, D., Perrot, M., Boucherie, H., and Jacquet, M. (1996) Microbiology 142, 459–467
11. Maillet, I., Lagniel, G., Perrot, M., Boucherie, H., and Labarre, J. (1996) J. Biol. Chem. 271, 10265–10270
12. Giertz, R. D., Schiedl, R. H., Willems, A. R., and Woods, R. A. (1995) Yeast 11, 355–360
13. Schmitt, M. E., Brown, T. A., and Trumpower, B. L. (1990) Nucleic Acids Res. 18, 3091–3092
14. Woychik, N. A., and Young, R. A. (1988) Mol. Cell. Biol. 9, 2834–2839
15. Godon, C., Lagniel, G., Lee, J., Buhler, J. M., Kieffer, S., Perrot, M., Boucherie, H., Toledano, M. B., and Labarre, J. (1998) J. Biol. Chem. 273, 22480–22489
16. Norbeck, J., and Blomberg, A. (1995) Electrophoresis 16, 149–156
17. Herrick, D., Parker, R., and Jacobson, A. (1990) Mol. Cell. Biol. 10, 2269–2284
18. Moskvina, E., Schuller, C., Maurer, C. T., Mager, W. H., and Ruis, H. (1998) Yeast 14, 1041–1050
19. Treger, J. M., Schmitt, A. P., Simon, J. R., and McKenzie, K. (1998) J. Biol. Chem. 273, 26875–26879
20. Caponigro, G., and Parker, R. (1996) Microbiol. Rev. 60, 233–249
21. Thompson, C. M., and Young, R. A. (1995) Proc. Natl. Acad. Sci. U. S. A. 92, 4587–4590
22. Parker, R., Herrick, D., Peltz, S. W., and Jacobson, A. (1991) Methods Enzymol. 194, 415–423
23. Collins, L. P., and Dawes, I. W. (1992) J. Gen. Microbiol. 138, 329–335
24. de Virgilio, C., Piper, P., Boller, T., and Wiemken, A. (1991) FEBS Lett. 288, 86–90
25. Lee, J., Godon, C., Lagniel, G., Spectre, D., Garin, J., Labarre, J., and Toledano, M. B. (1999) J. Biol. Chem. 274, 16040–16046
26. Schnell, N., Krems, B., and Entian, K. D. (1992) Curr. Genet. 21, 269–273
27. Lee, D. H., Sherman, M. Y., and Goldberg, A. L. (1996) Mol. Cell. Biol. 16, 4773–4781
28. Sanchez, Y., and Lindquist, S. L. (1990) Science 248, 1112–1115
29. Lindquist, S., and Kim, G. (1996) Proc. Natl. Acad. Sci. U. S. A. 93, 5301–5306
30. Elliott, B., Haltiwanger, R. S., and Putcher, B. (1996) Genetics 144, 923–933
31. Boy-Marcotte, E., Lagniel, G., Perrot, M., Busseareau, F., Boudsocq, A., Jacquet, M., and Labarre, J. (1999) Mol. Microbiol. 33, in press