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Camille Andrieu, Alexandra Vergnes, Laurent Loiseau, Laurent Aussel, Benjamin Ezraty. Characterisation of the periplasmic methionine sulfoxide reductase (MsrP) from Salmonella Typhimurium. Free Radical Biology and Medicine, 2020, 160, pp.506-512. 10.1016/j.freeradbiomed.2020.06.031. hal-02995688

HAL Id: hal-02995688 https://amu.hal.science/hal-02995688

Submitted on 10 Nov 2020 $\,$

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Free Radical Biology and Medicine



journal homepage: www.elsevier.com/locate/freeradbiomed

Short communication

Characterisation of the periplasmic methionine sulfoxide reductase (MsrP) from *Salmonella* Typhimurium



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ARTICLE INFO	A B S T R A C T
Keywords:	The oxidation of free methionine (Met) and Met residues inside proteins leads to the formation of methionine sulfoxide
Salmonella Typhimurium	(Met-O). The reduction of Met-O to Met is catalysed by a ubiquitous enzyme family: the methionine sulfoxide reductases
Protein oxidation Methionine sulfoxide reductase MsrP	(Msr). The importance of Msr systems in bacterial physiology and virulence has been reported in many species. Salmonella
	Typhimurium, a facultative intracellular pathogen, contains four cytoplasmic Msr. Recently, a periplasmic Msr enzyme
	(MsrP) has been identified in Escherichia coli. In the present study, the STM14_4072 gene from Salmonella was shown to
Molybdo-enzyme	encode the MsrP protein (StMsrP). We describe the experimental procedure and precautions for the production of this
	molybdo-enzyme. StMsrP was also demonstrated to reduce free Met-O and to catalyse the complete repair of an oxidized
	protein. More importantly, this study provides for the first time access to the exhaustive list of the Msr systems of a
	pathogen, including four cytoplasmic enzymes (MsrA, MsrB, MsrC, BisC) and one periplasmic enzyme (MsrP).

1. Introduction

Dioxygen is extremely reactive and can be transformed into reactive oxygen species (ROS) such as the superoxide anion (O2), hydrogen peroxide (H₂O₂) or the hydroxyl radical (HO⁻). ROS can react with other components present in their environment such as nitrogen or chlorine, leading to the formation of nitric oxide (NO) or hypochlorous acid (HOCl). These chemicals can then oxidize biomolecules such as proteins or nucleic acids. Therefore, ROS represent harmful weapons against living organisms which have evolved in aerobic environments over millennia, forcing them to adapt [1]. Their primary defence lines consist of antioxidant enzymes such catalases, superoxide dismutases or peroxidases, allowing the conversion or degradation of ROS. Their secondary defence lines are made up of enzymes involved in the repair of oxidized biomolecules, allowing them to restore their reduced state. Among these enzymes, thioredoxins (Trx) and glutaredoxins (Grx) reduce cysteine disulfide bonds [2], whereas methionine sulfoxide reductases (Msr) catalyse the reduction of methionine sulfoxide (Met-O) into methionine (Met) [3,4].

In *Escherichia coli*, MsrA and MsrB reduce cytoplasmic proteinbound Met-O whereas MsrC and BisC reduce free Met-O residues [5–8]. Interestingly, MsrA and BisC present a stereospecificity towards the Sform whereas MsrB and MsrC reduce the R-form. This chemical specificity is not shared by MsrP, a molybdo-enzyme located in the periplasm which can reduce the two isoforms [9–12]. Whereas the four cytoplasmic Msr require Trx to restore their catalytic activity, MsrP relies on MsrQ, an inner membrane protein, to transfer electrons from the respiratory chain or possibly through the cytoplasmic flavin reductase Fre [9,13].

Salmonella enterica serovar Typhimurium is a facultative intracellular pathogen that is associated with a wide range of infections in mammals, ranging from self-limiting gastroenteritis to severe systemic diseases. The establishment of infection depends on the ability of the pathogen to adapt to various environments and to resist different stresses, including ROS production. *msrA*, *msrC* and *bisC* were previously shown to be important for *Salmonella* virulence in macrophages and mice, whereas *msrB* was not found to play any major role [14,15]. More recently, *msrA* was also suggested to be important for *Salmonella* colonisation in chicken [16] but to date, the presence of a periplasmic Msr has not been reported in *Salmonella* Typhimurium.

In the present paper, we show that the STM14_4072 open reading frame, so-called *yedY*, encodes a periplasmic Msr (StMsrP) in *Salmonella*. This enzyme has been purified and found to reduce free and proteinbound Met-O into Met. Its catalytic activities were measured using Met-O and DMSO as substrates. StMsrP was shown to fully repair an oxidized protein containing (R- and S-) Met-O. Finally, we have constructed a Met⁻ mutant lacking MsrP and the four cytoplasmic Msrs (MsrA, MsrB, MsrC and BisC) and found this strain unable to use Met-O, but overexpression of *msrP* was shown to restore Met-O assimilation. Overall, these results demonstrate that in *Salmonella* Typhimurium, five Msr are involved in protein repair and Met-O homeostasis.

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https://doi.org/10.1016/j.freeradbiomed.2020.06.031 Received 12 June 2020; Accepted 17 June 2020

Available online 01 August 2020

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Fig. 1. YedY sequence conservation.

A) Schematic view of the MsrPQ systems in *Escherichia coli*. MsrP and MsrQ are represented in blue and red, respectively. Met and Met-O are represented by purple hexagons and yellow stars, respectively. B) Schematic view of the *msrP* and *msrQ* locus in *E. coli* and their homologous genes in *Salmonella* Typhimurium 14028 and LT2 strains. C) Amino acid sequence alignment of StYedY (STM14_4072) of *Salmonella* with MsrP homologues EcMsrP (*E. coli* P76342) and RsMsrP (*Rhodobacter sphaeroides* Q3IXZ5). Each sequence is numbered accordingly. Characteristic residues of this type of enzyme are indicated in red above the sequence. The consensus sequence is represented in white within black boxes. D) Schematic representation of the protein domain organisation, TAT domain and the oxidoreductase molybdenum cofactor (Mo-MPT) binding site are represented in blue and in salmon pink, respectively. Asn89 and Cys146 residues are spotted above by a black diamond.

2. Results

2.1. Sequence analysis of StYedY (STM14_4072)

YedY enzymes belong to the Sulfite Oxidase (SO) family [EC1.8.3.1], which contains a molybdenum cofactor. The physiological activity of these periplasmic enzymes requires their membrane-bound redox partner YedZ (Fig. 1A). In *E. coli, msrP* and *msrQ* genes (formerly *yedY* and *yedZ*) are organised in an operonic structure. This arrangement is conserved in *Salmonella* species and corresponds to the STM14_4072 (*yedY*) and STM14_4073 (*yedZ*) genes (Fig. 1B). The

protein sequence comparison between YedY from *Salmonella* (StYedY) and fully characterized MsrP shows a percentage identity of 88% and 58% with EcMsrP from *E. coli* and RsMsrP from *Rhodobacter sphaeroides*, respectively (Fig. 1C). The first 44 residues of the StYedY protein correspond to the TAT signal peptide containing the distinctive "twin-arginine" motif and the recognition site for signal peptidase (AXA). The residues involved in the binding of molybdenum (Cys146) and molybdopterin (MPT) are highly conserved. Moreover, the well-documented asparagine residue of the YedY enzyme family [17,18] is found in StYedY (Asn89) (Fig. 1C and D). Finally, analysis of the StYedY sequence suggests that this enzyme should belong to the MsrP family.



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Fig. 2. Purification of recombinant StYedY.

A) SDS-PAGE protein profile showing overexpression and the first step of purification of StYedY-6His by Ni-NTA affinity chromatography. L = protein ladder; Lane 1: protein extract before induction; Lane 2: protein extract after induction; Lane 3: pellet fraction after periplasm preparation; Lane 4: Periplasmic extract; Lane 5: flow through; Lanes 6 to 10: protein elution by imidazole; Lane 11: pooled fractions. Precursor and mature forms of StMsrP (formerly StYedY) are represented by dotted lines. B) SDS-PAGE protein profile showing the second purification step of StYedY by size exclusion chromatography Superdex 75 10/300. L = protein ladder; Lane 1: same as Lane 11 panel A; Lanes #18 to #30 represent the fraction number of the exclusion chromatography column. The mature form of StMsrP is represented (33.6 kDa).

2.2. Purification of StYedY

To examine the catalytic properties of StYedY, the protein carrying a C-terminal Histidine tag was purified. As YedY is a molybdenum cofactor enzyme, we did not use the classic E. coli BL21 (DE3) strain because the insertion site of the DE3 prophage is located within the molybdate uptake genes [19]. Thus, we used the specific TP1004 strain which contains a deletion of Mo-bis (MGD) synthesis genes ($\Delta mobAB$) and therefore accumulates Mo-MPT [19]. As shown in Fig. 2A, the induction of pAC001 results in the production of a high amount of the precursor form of StYedY (38.3 kDa). The periplasmic fraction was isolated and loaded onto a Ni-NTA affinity chromatography to purify the mature form (33.6 kDa). Despite this precaution, a slight contamination by the precursor form was observed. Therefore, the different fractions were pooled, concentrated and passed through a size exclusion chromatography column. Proteins were eluted with an apparent molecular weight corresponding to the mature monomeric form. This step allowed us to obtain a pure fraction of mature protein StYedY.

2.3. StMsrP (STM14_4072) reduces free Met-O and DMSO

Next, we determined the kinetic parameters of StYedY using benzyl viologen (BV) as an artificial electron donor and free Met-O or DMSO as substrates. The initial reaction rates were plotted as a function of substrate concentrations and the non-linear regression of the Michaelis-Menten equation was calculated (Fig. 3A). StYedY was shown to reduce free Met-O and DMSO. Using the free Met-O, a kcat of $\sim 17.5 \text{ s}^{-1}$ and a Km of $\sim 15 \text{ mM}$ were determined, yielding a catalytic efficiency (kcat/Km) of $\sim 1 \text{ mM}^{-1} \text{ s}^{-1}$. With DMSO, the kcat and a Km values were found to be $\sim 38 \text{ s}^{-1}$ and $\sim 39 \text{ mM}$, respectively, yielding a catalytic efficiency (kcat/Km) of $\sim 1 \text{ mM}^{-1} \text{ s}^{-1}$. Thus, StYedY, RsMsrP and EcMsrP exhibit a similar ability to reduce free Met-O and DMSO [10,11]. From now on, the protein encoded by *yedY* (STM14_4072) will be referred to as StMsrP for MsrP of *Salmonella* Typhimurium.

2.4. In vivo evidence for methionine sulfoxide reductase activity of StMsrP

Met-O can be used as a Met source in all living organisms [20–22]. This property is strictly dependent on Msr activity [9]. We used this point to test the capacity of StMsrP to reduce Met-O *in vivo*. We first constructed a *Salmonella* Met auxotroph mutant lacking all cytoplasmic Msrs (MsrA, MsrB, MsrC and BisC) and found this strain (*met* $\Delta 4msr^{cyto}$) able to use partially Met-O as the Met source. We only observed small colonies after 5 days of incubation (Fig. 3B). Interestingly, deletion of *msrP* in the *met* $\Delta 4msr^{cyto}$ background prevented the strain to grow on Met-O. Overproduction of StMsrP using the pAC001 plasmid in the *met* $\Delta 4msr^{cyto}$ background restored growth on Met-O (Fig. 3B). From this experiment, we can conclude that StMsrP reduces free Met-O *in vivo*, suggesting that no extra Met-O reductase enzymes are present in *Salmonella* Typhimurium.

2.5. StMsrP leads to complete repair of an oxidized protein containing Met-O

EcMsrP has been shown to reduce protein-bound Met-O. Remarkably, the lack of diastereospecificity of MsrP confers a specific role to this oxidoreductase. Consequently, full repair of an oxidized protein in the periplasm requires only the action of MsrP while in the cytoplasm, it requires both MsrA and MsrB. First, we assessed the capacity of StMsrP to reduce protein-bound Met-O using the oxidized form of calmodulin (CaMox) as a substrate. Gel shift assays were used to detect Met-O residues in the protein. Indeed, the Met-O-containing proteins migrate slower than their reduced counterparts as observed by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) (Fig. 4). Exposure of CaMox to StMsrP restored its initial migration level, suggesting that StMsrP can reduce Met-O residues in CaMox (Fig. 4A). Co-incubation with an artificial reducing system is necessary for this activity. Our results also show that the incubation of CaMox with StMsrP led to a migration of the protein to the same position that reduced CaM, as well as CaMox treated with EcMsrP or with MsrA plus MsrB (Fig. 4B). In the

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Fig. 3. StMsrP reduces free Met-O. A) StMsrP activities with Met-O and DMSO. The StMsrP activities were measured using benzyl viologen (BV) as an artificial electron donor under anaerobic condition. BV was initially reduced with sodium dithionite and oxidation was monitored at 600 nm after addition of the enzyme. Data presented are the average of three individual experiments ± S.D. The Michaelis-Menten parameters were determined using Graphpad Prism software. Met-O: free methionine sulfoxide; DMSO: dimethyl sulfoxide. B) The $\Delta metA \Delta 4msr^{cyto}$ (msrA, msrB, msrC and bisC) and the Δ metA Δ 4msr^{cyto} Δ msrP were spread on M9 plates or M9 supplemented with Met or Met-O. The $\Delta metA \Delta 4msr^{cyto} \Delta msrP$ strains carrying the empty or the pAC001 vector (overexpressing msrP) were spread on M9 plates or M9 supplemented with Met or Met-O (in presence of ampicillin and IPTG). The plates were incubated at 37 °C for 5 days and scanned.

control, exposure of CaMox to MsrA or MsrB led to a migration approximately halfway between the fully reduced and oxidized forms of the CaM, suggesting a partial repair of the protein. Altogether, these results demonstrate that StMsrP fully repairs oxidized proteins containing Met-O, which strongly suggests the absence of diastereospecificity of this enzyme as previously shown for its homologues in *E. coli* and *R. sphaeroides*.

3. Discussion

The universal methionine sulfoxide reductase enzyme family provides defence against oxidative stress by reducing protein-bound Met-O into Met and maintaining the free Met pool in a reduced state. The importance of these reducing systems in bacterial physiology and virulence has been reported in almost all bacteria in which their roles have been studied. Therefore, the characterization of all Msr activities within an organism is essential to have a global view of this process. Cytoplasmic enzymes (MsrA, MsrB, MsrC and BisC) have been widely studied in *Salmonella* Typhimurium, but the putative periplasmic methionine sulfoxide reductase (MsrP), recently discovered in *E. coli*, has not been characterized so far. In the present study, we identified the *yedY* gene from *Salmonella* as encoding the StMsrP protein. We have precisely described the experimental procedure for producing MsrP in order to avoid the common mistake, which would consist in using the *E. coli* BL21 (DE3) strain. This protocol was not sufficiently highlighted in our previous publication [9], leading to unsuccessful purification of such enzymes in other laboratories.

StMsrP was purified, characterized and found to reduce free and protein-bound Met-O. The catalytic activities measured are similar to



Fig. 4. StMsrP reduces protein-bound Met-O. A) SDS-PAGE protein migration showing repair of CaMox by StMsrP. Oxidation of Met within calmodulin (CaM) led to a mobility shift of the oxidized protein (CaMox). CaMox was incubated with StMsrP with or without an electron donor system. B) SDS-PAGE protein migration showing CaMox fully repaired by StMsrP. To visualize partial repair of CaMox, we used the diastereospecific enzymes MsrA or MsrB. Treatment of CaMox with StMsrP, EcMsrP or MsrA plus B led to the same migration profile.

Table 1				
List of strains	used	in	this	study

those reported for EcMsrP and RsMsrP [10,11]. However, the efficiency difference observed could be explained by the presence of a Cter (His) tag in our enzyme, as previously reported by Sabaty and collaborators [23]. Importantly, we report that StMsrP leads to complete repair of an oxidized protein, suggesting that this reductase is non-stereospecific, like EcMsrP and RsMsrP. Another significant finding from this study is the description of the exhaustive list of Msr's activities in *Salmonella*. By showing that *met* Δ *4msr*^{cyto} Δ *msrP* strain was unable to growth on Met-O, we have demonstrated that no other Met-O reductase activities were present in *Salmonella*. Therefore, we can conclude for the first time that *Salmonella* Typhimurium contains four cytoplasmic (MsrA, MsrB, MsrC and BisC) and one periplasmic (MsrP) methionine sulfoxide reductase enzymes.

Finally, additional studies will be required to address the involvement of StMsrP in the virulence of *Salmonella*, as already reported for its homologous protein in *Campylobacter Jejuni* [24].

4. Materials & methods

4.1. Bacterial strains and plasmids

The strains and primers used in this study are listed in Table 1 and Table 2, respectively.

Salmonella Typhimurium ATCC14028 was used as a wild-type strain. Deletions of genes were carried out using one-step λ Red recombinase chromosomal inactivation system [25]. Deletions were transferred to the wild-type strain using P22 transduction procedures and verified by PCR. In order to build the StMsrP-His6 expression vector (pAC001), the STM14_4072 gene was amplified using *StmsrP_EcoRLATG_Fw* and *StmsrP-His6_STOP_Hind*III_*Rv* primers. The PCR product was cloned into pJF119EH using *Eco*RI and *Hind*III restriction sites. The construction was verified by DNA sequencing.

4.2. Protein purification

The StMsrP-His6 purification was carried out as follows: TP1004 cells harboring plasmid pAC001 were grown aerobically at room temperature in terrific broth supplemented with sodium molybdate (1.5 mM) and ampicillin (100 μ g/mL). When cells reached an OD_{600nm} = 0.6, 0.1 mM IPTG was added for 15 h. Cells were centrifuged (3,000 g, 30 min, 4 °C). Pellets were washed with PBS and gently resuspended in TBE (Sucrose 0.5 M, Tris-HCl 0.2 M pH 8, EDTA 1 mM). After 30 min in ice, suspensions were centrifuged (13,000 g, 20 min, 4 °C). Imidazole (25 mM) was added to the supernatant and loaded onto a HisTrap HP column (GE Healthcare) equilibrated with buffer A (PBS, imidazole 25 mM). After washing the column with 10 % of buffer B (PBS, imidazole 500 mM), StMsrP-His6 was eluted by applying 50 % of buffer B. The fractions containing StMsrP-His6 were

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Strain	Genotype and description	Source			
14028	14028 WT	Laboratory collection			
$\Delta msrA$	14028 $\Delta msrA::Kan^r$	Laboratory collection			
$\Delta msrB$	14028 $\Delta msrB::Cm^r$	Laboratory collection			
$\Delta msrC$	14028 $\Delta msrC::Cm^r$	Laboratory collection			
$\Delta bisC$	14028 ∆bisC::Kan ^r	Laboratory collection			
$\Delta msrP$	14028 $\Delta msrP$::Kan ^r	Laboratory collection			
$\Delta metA$	14028 $\Delta metA::Cm^r$	This study			
$\Delta metA \Delta 4 msr^{cyto}$	14028 $\Delta msrA \Delta msrB \Delta msrC \Delta bisC \Delta metA::Cm^{r}$	This study			
$\Delta metA \Delta 4 msr^{cyto} \Delta msrP$	14028 $\Delta msrA \Delta msrB \Delta msrC \Delta bisC \Delta metA::Cm^{r}$	This study			
$\Delta metA \Delta 4 msr^{cyto} \Delta msrP$ + empty vector	14028 Δ <i>msrA</i> Δ <i>msrB</i> Δ <i>msrC</i> Δ <i>bisC</i> Δ <i>msrP</i> Δ <i>metA</i> ::Cm ^r /pJF119EH	This study			
$\Delta metA \ \Delta 4msr^{cyto} \ \Delta msrP + pStMsrP$	14028 $\Delta msrA \Delta msrB \Delta msrC \Delta bisC \Delta msrP \Delta metA::Cmr/pAC001$	This study			
Escherichia coli TP1004	RK4353 <i>∆mobAB</i> ::Kan ^r	Received from Tracy Palmer			

Table 2

List of primers used in this study.

Name	Sequence (5' to 3')	Used for
Name msrA_start msrA_stop msrB_start msrB_stop msrA100_Fw msrA_+100_Rv msrB100_Fw msrB100_Fw	Sequence (5' to 3') ATGAGTTTATTTGATAAAAAACATCTGGTTACTCAAGCAGGTGTAGGCTGGAGCTGCTTC TCACGCGTCAGGCGGCAGGCAAACGCCGATCCCGCCAATCCATATGAATATCCTCCTTAG GTGAGATGTGAGCAAATGGCTAACCAACCTTCAGCAGAAGGTGTAGGCTGGAGCTGCTTC TCAGCCTTTCAGTTGATCGCCGTTTTTTCTCGTCAGAAAAGCATATGAATATCCTCCTTAG TTTCTTATGGTTAAATCCAGA ATCCTCGGAAGGGAAG	Used for Strain construction Strain construction Strain construction Strain construction Deletion verification Deletion verification Deletion verification
msrD ₂ + 100_Rv msrP ₂ -100_Fw msrD ₂ + 100_Rv msrC ₂ -100_Fw msrC ₂ + 100_Rv bisC ₂ -100_Fw metA ₂ -100_Fw metA ₂ + 100_Rv StmsrP ₂ EcoRI _A TG ₂ Fw StmsrP ₂ His6 ₂ STOP ₂ HindIII_Rv	AAAATTTACACTTAATTAAC CACCGTGATTTATCGCCCAAA CTAATTGACCTGATCGTTAGC CATGAAATTTCCTGATTACAA TATGACGTCTGCACATGAAGA GTTTACCAGAAAATCAATCC TCACCTTGGACTTGCAGACTC ATTTAATTACATGATGAGAGTG GCGCCCGAATTCATGAAAAAGATACGTCCATTACAG GAAGAAAAGCTTTTAGTGGTGGTGGTGGTGGTGGGCAGCAAAATTCTCCCCGCAAATTGAG	Deletion verification Deletion verification Deletion verification Deletion verification Deletion verification Deletion verification Deletion verification Plasmid construction Plasmid construction

pooled, concentrated and loaded on GF Superdex 75 10/300 GL. Purification of EcMsrP-His6, calmodulin, EcMsrA and EcMsrB were carried out as described previously [6,9].

4.3. Kinetic analysis of StMsrP-His6 activity

The StMsrP-His6 reductase activity was measured as described previously (Gennaris, 2015). 25 nM and 16 nM of StMsrP-His6 were used for the measurement of activity on Met-O and DMSO respectively.

4.4. In vivo Met-O assimilation assay

 $\Delta metA \ \Delta 4msr^{cyto}$ and $\Delta metA \ \Delta 4msr^{cyto} \ \Delta msrP$ were streaked onto M9 minimal medium plates supplemented with Met or Met-O (20 µg mL⁻¹). Plates were incubated at 37 °C for 5 days. $\Delta metA \ \Delta 4msr^{cyto} \ \Delta msrP + empty vector and \ \Delta metA \ \Delta 4msr^{cyto} \ \Delta msrP + pAC001$ were streaked onto M9 minimal medium plates supplemented with ampicillin (30 µg mL⁻¹), IPTG (0.1 mM) and either Met or Met-O (20 µg mL⁻¹). Plates were incubated at 37 °C for 5 days.

4.5. In vitro repair of oxidized CaM

Oxidation of calmodulin (CaM) and repair of oxidized CaM (CaMox) by MsrP-His6 or MsrA/B were carried out as described previously [9].

Acknowledgements

We thank all the members of the Ezraty group for comments on the manuscript, advice and discussion throughout the work. We thank Nassos Typas (EMBL-Heidelberg) and Tracy Palmer (Newcastle University) for providing strains, and Rebecca Stevens (GAFL, INRAE Avignon) for critical reading of the manuscript. This work was supported by the Fondation pour la Recherche Médicale, grant number ECO201806006837, to Andrieu Camille. This work was supported by grants from the Agence Nationale de la Recherche (ANR) (#ANR-16-CE11-0012-02 METOXIC) and the Centre National de la Recherche Scientifique (CNRS) (#PICS-PROTOX). We also thank J.F. Collet, L. Tarrago, Y. Cherrak, A. Battesti and the Py group for helpful suggestions and comments on this work.

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